

Published in final edited form as:

Hepatology. 2011 October ; 54(4): 1157–1166. doi:10.1002/hep.24541.

HLA-B27 Selects for Rare Escape Mutations that Significantly Impair Hepatitis C Virus Replication and Require Compensatory Mutations

Christoph Neumann-Haefelin^{1,2,3,*}, Cesar Oniangue-Ndza^{1,*}, Thomas Kuntzen^{1,5,*}, Julia Schmidt^{2,3,4}, Katja Nitschke^{2,4}, John Sidney⁶, Célia Caillet-Saguy⁷, Marco Binder⁸, Nadine Kersting², Michael W. Kemper¹, Karen A. Power¹, Susan Ingber⁹, Laura L. Reyor¹⁰, Kelsey Hills-Evans¹⁰, Arthur Y. Kim¹⁰, Georg M. Lauer⁹, Volker Lohmann⁸, Alessandro Sette⁶, Matthew R. Henn⁹, Stéphane Bressanelli⁷, Robert Thimme², and Todd M. Allen^{1,#}

Christoph Neumann-Haefelin: christoph.neumann-haefelin@uniklinik-freiburg.de; Cesar Oniangue-Ndza: coniangue-ndza@partners.org; Thomas Kuntzen: t.kuntzen@gmx.de; Julia Schmidt: julia.schmidt@uniklinik-freiburg.de; Katja Nitschke: katja.nitschke@uniklinik-freiburg.de; John Sidney: jsidney@liai.org; Célia Caillet-Saguy: celia.caillet@vms.cnrs-gif.fr; Marco Binder: marco_binder@med.uni-heidelberg.de; Nadine Kersting: nadine.kersting@uniklinik-freiburg.de; Michael W. Kemper: earthmk@umich.edu; Karen A. Power: kapower@partners.org; Susan Ingber: susanzellsingber@gmail.com; Laura L. Reyor: lreyor@partners.org; Kelsey Hills-Evans: khills-evans@partners.org; Arthur Y. Kim: akim1@partners.org; Georg M. Lauer: glauer@helix.mgh.harvard.edu; Volker Lohmann: volker_lohmann@med.uni-heidelberg.de; Alessandro Sette: alex@liai.org; Matthew R. Henn: mhenn@broadinstitute.org; Stéphane Bressanelli: stephane.bressanelli@vms.cnrs-gif.fr; Robert Thimme: robert.thimme@uniklinik-freiburg.de; Todd M. Allen: tallen2@partners.org

¹Ragon Institute of MGH, MIT and Harvard, Boston, MA, USA ²Department of Medicine II, University of Freiburg, Freiburg, Germany ³Centre of Chronic Immunodeficiency (CCI), University of Freiburg, Freiburg, Germany ⁴Faculty of Biology, University of Freiburg, Freiburg, Germany ⁵Department of Gastroenterology and Hepatology, University Hospital Zurich, Zurich, Switzerland ⁶La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA ⁷Laboratoire de Virologie Moléculaire et Structurale, CNRS UPR3296, Centre de Recherche de Gif, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France ⁸Department of Infectious Diseases, Molecular Virology, University of Heidelberg, Germany ⁹Gastrointestinal Unit and ⁸ Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, MA USA ¹⁰Broad Institute, Cambridge, MA, USA

Abstract

HLA-B27 is associated with spontaneous viral clearance in hepatitis C virus (HCV) infection. Viral escape within the immunodominant HLA-B27 restricted HCV-specific CD8+ T cell epitope NS5B₂₈₄₁₋₂₈₄₉ (ARMILMTHF) has been shown to be limited by viral fitness costs as well as broad T cell cross-recognition, suggesting a potential mechanism of protection by HLA-B27. Here, we studied the subdominant HLA-B27 restricted epitope NS5B₂₉₃₆₋₂₉₄₄ (GRAAICGKY) in order to further define the mechanisms of protection by HLA-B27. We identified a unique pattern of escape mutations within this epitope in a large cohort of HCV genotype 1a infected patients. The predominant escape mutations represented conservative substitutions at the main HLA-B27 anchor residue or a T cell receptor contact site, neither of which impaired viral replication capacity as assessed in a subgenomic HCV replicon system. In contrast, however, in a subset of HLA-B27+ subjects rare escape mutations arose at the HLA-B27 anchor residue R₂₉₃₇, which nearly abolished viral replication. Notably, these rare mutations only occurred in conjunction with the

#Corresponding author: Todd M. Allen, MGH-East, CNY 6625, Bldg 149 13th Street, Charlestown, MA 02129, Phone: (617) 726-7846, Fax: (617) 724-8586, tallen2@partners.org.
*equal contribution

selection of two equally rare, and structurally proximal, upstream mutations. Co-expression of these upstream mutations with the rare escape mutations dramatically restored viral replication capacity from <5% to ≥70% of wild-type levels.

Conclusion—The selection of rare CTL escape mutations in this HLA-B27 restricted epitope dramatically impairs viral replicative fitness unless properly compensated. These data support a role for the targeting of highly-constrained regions by HLA-B27 in its ability to assert immune control of HCV and other highly variable pathogens.

Keywords

Hepatitis C virus; T cell response; viral escape; HLA-B27; viral fitness

The HLA class I allele B27 has a protective role in both human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections (1–2), two of the most highly variable pathogens for which effective vaccines are still lacking. In HIV infection, HLA-B27 is associated with low viral loads, slow CD4+ decline, and the delayed onset of AIDS (1). Protection by HLA-B27 has been linked to the immunodominant targeting of a highly conserved CD8+ epitope in Gag which requires a complicated pathway of viral escape to evade this response. Escape from this B27-KK10 response requires a multitude of mutations. First, an L₂₆₈M mutation inside the epitope impairs T cell recognition and also impairs dendritic cell (DC) function through increased interaction with an inhibitory receptor (3). A subsequent R₂₆₄K mutation at the main HLA-B27 binding anchor of the epitope, which achieves more effective CTL escape by abolishing epitope presentation, typically occurs much later in infection and is associated with disease progression (3). The late appearance of the main R₂₆₄K escape mutation can be attributed to its substantial impact on viral replicative fitness when expressed alone, and thus it is commonly associated *in vivo* with a S₁₇₃A compensatory mutation located 90 amino acids upstream of the epitope (3–5). Thus, the ability of HLA-B27 to impart substantial and durable control of HIV is due in part to the requirement for a compensatory mutation to enable effective CTL escape from this dominant immune response.

In HCV genotype 1 infection, the majority of HLA-B27+ individuals are able to clear the virus spontaneously (2, 6). We have previously described several HLA-B27 restricted HCV-specific CD8+ T cell epitopes (7). One of these epitopes, NS5B₂₈₄₁₋₂₈₄₉ (ARMILMTHF), is particularly immunodominant since it is recognized in nearly all HLA-B27+ individuals with resolved HCV genotype 1 infection. In addition, nearly all HLA-B27+ patients who progress to chronic HCV genotype 1 infection display viral escape mutations in this epitope, suggesting that this response exerts significant selection pressure (7). Notably, this immunodominant HLA-B27 restricted CD8+ epitope is only present in HCV genotype 1 but absent from the other HCV genotypes, possibly explaining why HLA-B27+ individuals are not protected from chronic non-genotype 1 HCV infection (8). Importantly, viral escape within this epitope does not occur easily. Mutations experimentally introduced in replicon constructs at the HLA-B27 binding anchor residues of the epitope exhibit high fitness costs *in vitro*, and have therefore not been found in viral sequences derived from patient specimens. In contrast, while mutations at T cell receptor contact residues within the epitope are tolerated by the virus, broad cross-recognition of these escape variants requires multiple ‘clusters’ of these mutations to achieve efficient escape from this CD8+ T cell response (9). The requirement for clustered mutations to escape from dominant HLA-B27 restricted CD8+ T cell responses in both HIV and HCV infections suggests that HLA-B27 may be achieving immune control of these highly variable pathogens through the targeting of critical regions of these viruses in which viral escape is impaired.

To further explore this hypothesis, here we have analyzed the patterns of viral escape in a second HLA-B27 restricted CD8 epitope NS5B₂₉₃₆₋₂₉₄₄ (GRAAICGKY). This CD8+ T cell response also selects for viral escape mutations in the majority of patients with chronic HCV genotype 1a infection. We identified a subset of escape mutations within the epitope that nearly abolished viral replication, and thus required additional, compensatory mutations located upstream of the epitope. This was in striking similarity to the escape pathways described for the immunodominant HLA-B27 restricted KK10 epitope in HIV Gag (3–4), providing unique insight into the mechanisms by which some HLA alleles may be mediating their protective effect over HCV and other highly variable pathogens.

Experimental procedures

Patients

Viral sequences from 404 mostly (>90%) treatment-naïve patients with chronic HCV genotype 1a infection were obtained previously (10)(Kuntzen et al., manuscript in preparation). HLA class I typing was performed using standard molecular typing. 19 of the 404 patients were positive for HLA-B27 (supplementary table S1). In addition, PBMCs were obtained from 14 HLA-B27+ patients with chronic HCV genotype 1a infection (10 patients from the above mentioned cohort and 4 patients enrolled at the Freiburg University Medical Center) as well as from 2 HLA-B27+ patients with acute-resolving genotype 1a infection enrolled at the Freiburg University Medical Center. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review boards at Massachusetts General Hospital, Boston, and the University of Freiburg, respectively. Written informed consent was obtained from each study participant.

Plasmid construction, electroporation, transient-replication assays, NS5B structure analyses, cellular T cell assays and HLA-B2705 peptide binding assay

These methods are described in the supplementary methods section.

Statistics

Frequencies of substitutions in HLA-B27+ and HLA-B27- patients (Table 1) were compared using two-tailed Fisher's exact test (<http://www.graphpad.com/quickcalcs>). Replicative capacities of mutants and wild-type (Fig. 2 and 4, supplementary Fig. S1) were compared using paired t test (GraphPad Prism 5.0). P values <0.05 were considered significant.

Results

CTL escape in the B27-GRAAICGKY epitope typically occurs through conservative mutations

In a large cohort analysis of 404 full-length HCV genotype 1a sequences, we identified that viral escape in the HLA-B27 restricted CD8+ T cell epitope NS5B₂₉₃₆₋₂₉₄₄ (GRAAICGKY) was comprised of a unique array of CTL escape mutations. In the 19 HLA-B27+ individuals the predominant escape mutations within this epitope arose at either the main B27 anchor residue (R₂₉₃₇K) or at a T cell receptor contact site (I₂₉₄₀T) (Fig. 1). In each case, these mutations represented conservative substitutions with the swapping of positively charged residues at R₂₉₃₇K, and the substitution of uncharged residues at I₂₉₄₀T. Notably, however, in two B27+ subjects more rare escape mutations were observed, with R₂₉₃₇ replaced by the uncharged amino acids serine (R₂₉₃₇S) or glycine (R₂₉₃₇G) (Fig. 1). In addition, in one B27-positive subject the I₂₉₄₀ residue was replaced by a rare valine (I₂₉₄₀V) substitution in conjunction with the R₂₉₃₇K mutation (Fig. 1). Due to the more frequent development of the R₂₉₃₇K and I₂₉₄₀T mutations we hypothesized that these more conservative substitutions

might only minimally impact viral replication (viral fitness), while the more rare mutations might be associated with higher fitness costs.

Common escape mutations within the B27-GRAAICGKY epitope have no effect on viral replication

To examine the impact of these CTL escape mutations on the virus we constructed subgenomic HCV replicons bearing the different viral escape mutations. Replicative capacities of the mutants were then measured in a transient *in-vitro* replication system in hepatoma cells using the luciferase reporter gene as a marker for HCV replication. As predicted, no significant differences in the replication capacity of either of the constructs containing the frequent mutations R₂₉₃₇K or I₂₉₄₀T were observed as compared to the parental 'wild-type' construct (Fig. 2). In fact, in this *in vitro* assay, the two variants even exhibited a slight, though not statistically significant, increase in replication. This unimpaired replication is in agreement with the observation that both mutations are more frequently observed in B27-negative subjects, with R₂₉₃₇K being the dominant viral species in 21/385 B27-negative patients (5.5%) and I₂₉₄₀T representing the dominant viral species in 16/385 B27-negative patients (4.2%) (Table 1).

Rare escape mutations within the B27-GRAAICGKY epitope substantially impair viral replication

In contrast to the common R₂₉₃₇K HLA-B27 anchor binding mutation, both of the rare R₂₉₃₇S and R₂₉₃₇G mutations nearly completely abolished viral replication (Fig. 2), including the R₂₉₃₇G mutation in conjunction with I₂₉₄₀T as observed in one patient (Fig. 1). The greater impact of these two rare escape mutations on viral replication is also in agreement with the absence of these mutations in HLA-B27 negative individuals; indeed, the R₂₉₃₇S mutation was not found in any of the 385 B27-negative patients in the cohort, while the R₂₉₃₇G mutation was only observed in a single B27-negative patient (0.3%; Table 1). In addition, the I₂₉₄₀V mutation, alone or in combination with mutation R₂₉₃₇K, was also found to impair viral replication, resulting in reductions in replication of 15% and 24%, respectively, although these results did not reach statistical significance. Considering that the I₂₉₄₀V substitution is only rarely observed in B27-negative patients (1/385 patients, 0.3%; Table 1) suggests that even this moderate reduction in replication capacity may have a significant effect on the virus *in vivo* analogous to the impact of the M184V drug-resistance mutation in HIV (11).

Mutations at position E₂₈₇₅ and P₂₈₈₁ co-evolve with rare escape mutations at positions R₂₉₃₇ and I₂₉₄₀

The observation that mutations R₂₉₃₇S and R₂₉₃₇G nearly completely impair viral replication was surprising given their presence in subjects demonstrating significant viral loads (e.g., the B27-positive patient with mutations R₂₉₃₇G/I₂₉₄₀T had a viral load of 33.9×10^6 IU/ml, and the B27-negative patient with mutation R₂₉₃₇G had a viral load of 22.5×10^6 IU/ml). We therefore hypothesized that these mutations might occur in conjunction with one or several additional mutations that function as compensatory mutations to alleviate their high costs to viral fitness. In screening our cohort of 404 HCV genotype 1a infected patients for additional substitutions co-evolving with the rare HLA-B27 associated mutations R₂₉₃₇G or R₂₉₃₇S throughout the HCV polyprotein, we found co-evolution (defined as a mutation present in at least 2 of the three patients with the R₂₉₃₇G or R₂₉₃₇S mutation but not frequently occurring in other patients) at only two amino acid residues, E₂₈₇₅ and P₂₈₈₁ (Fig. 3). These two residues do neither overlap with previously reported B27-restricted epitopes nor with predicted B27-restricted epitopes (T cell epitope prediction algorithm: www.iedb.org).

Residue E₂₈₇₅ was found to be highly conserved, with 396 of 404 patients (98.0%) displaying the consensus residue at this position (Table 1). Only 8 patients carried sequence variations at this position: 4 B27-negative patients (0.8%) exhibited an E₂₈₇₅Q substitution, while this substitution did not occur in HLA-B27+ patients (not significant). Conversely, however, a substitution of the negatively charged amino acid glutamate by the positively charged amino acid lysine (E₂₈₇₅K) occurred in three of the 19 HLA-B27+ patients (15.8%), notably more frequent than in the 0.3% (1/385) of B27-negative subjects (P=0.0003, Table 1). Strikingly, as shown in Figure 3A, all four E₂₈₇₅K substitutions occurred together with one of the rare mutations at position R₂₉₃₇ (R₂₉₃₇S or R₂₉₃₇G) or I₂₉₄₀ (I₂₉₄₀V). The co-expression of these very rare mutations indicated that E₂₈₇₅K may indeed be required for the development of these rare escape mutations, functioning to compensate for their impacts on viral replication. This hypothesis is confirmed by structural analysis of genotype 1a NS5B. As shown in Figure 3B, R₂₉₃₇'s positively charged side chain inserts between three negatively charged side chains, of which the central one is that of E₂₈₇₅. Mutating R₂₉₃₇ to either S (Figure 3C) or G (not shown) leaves a cluster of three negative charges, an unfavorable configuration that is compensated by the single E₂₈₇₅K mutation (Figure 3C). Still, these changes would affect the conformation of the so-called 'beta-flap', a flexible beta-structure in NS5B's 'thumb' domain at the base of which are the three negatively charged residues (Figure 3B). The beta-flap is one of two main anchors of the 'linker', a structural element whose movement has been proposed to be critical for NS5B's enzymatic activity (12). The other anchor is made by the outer faces of two helices in the thumb, harboring residue 2881 and the NS5B₂₉₃₇₋₃₉₄₄ epitope, respectively. P₂₈₈₁ in particular is sandwiched between two hydrophobic residues of the linker, an interaction that is changed by mutation to the bulkier L (not shown) or to the more polar Q (Figure 3C). Substitutions at position 2881 are more commonly observed than at position 2875, with 36 of 404 patients (8.9%) showing a substitution of the consensus residue proline (P) (Table 1), however, this substitution is also associated with HLA-B27 (5/19 HLA-B27+ patients [26.3%] compared to 31/385 HLA-B27-patients [8.1%], P=0.0183). Notably, three out of the 5 substitutions in B27-positive patients occurred in those three patients with rare mutations at positions R₂₉₃₇ or I₂₉₄₀ (Figure 3A).

Mutations at positions E₂₈₇₅ and P₂₈₈₁ restore the fitness defects of rare escape mutations in the B27-GRAAICGKY epitope

In order to directly test whether the observed mutations at positions E₂₈₇₅ and P₂₈₈₁ can compensate for the impact on viral replication of the rare mutations at positions R₂₉₃₇ or I₂₉₄₀, we co-expressed the different mutations in the subgenomic replicon system. As shown in Figure 4, co-expression of mutations R₂₉₃₇G, R₂₉₃₇G/I₂₉₄₀T, or R₂₉₃₇S with mutation E₂₈₇₅K led to a marked, though not complete restoration of viral replication to levels of 39%, 28%, and 80% compared to wild-type, respectively. The same was true for co-expression of the three respective R₂₉₃₇ mutations with mutations at P₂₈₈₁ (P₂₈₈₁L or P₂₈₈₁Q, respectively), resulting in viral replication levels of 17–31% compared to wild-type. Only the combined co-expression of the position R₂₉₃₇ mutations together with both candidate compensatory mutations (E₂₈₇₅K and P₂₈₈₁L/Q) led to a nearly complete restoration of viral replication, with replication levels of approximately 70% in the case of mutations R₂₉₃₇G and R₂₉₃₇G/I₂₉₄₀T and ≥100% in the case of mutation R₂₉₃₇S (Fig. 4). These results show clearly that the HLA-B27 associated substitutions at positions E₂₈₇₅ and P₂₈₈₁ are indeed compensatory mutations for the strong replicative defects caused by the substitution of arginine at R₂₉₃₇ by Glycine or Serine. When tested alone, the E₂₈₇₅K and P₂₈₈₁Q mutations were associated with a slightly reduced replication level (~77% and 92% of wild-type, respectively) as compared to wild-type, explaining why these substitutions occur rarely in B27-negative patients (1/385 patients [0.3%] and 5/385 patients [1.3%], respectively, Table 1). The subgenomic replicon carrying the P₂₈₈₁L mutation alone,

however, replicated at levels that even exceed the wild-type replication level. This finding may at least partially be due to the *in vitro* system used here. However, it is in agreement with the observation that the P₂₈₈₁L mutation is rather frequently observed in B27-negative patients *in vivo* (24/385 patients [6.2%], Table 1).

Escape mutations associated with a high viral fitness cost may be required for successful escape from the B27-GRAAICGKY response in some patients

Considering that the rare mutations R₂₉₃₇S, R₂₉₃₇G, and R₂₉₃₇G/I₂₉₄₀T require a complicated pathway of compensatory mutations, it is surprising that these mutations nevertheless occur in a subset of HLA-B27-positive patients. This finding led us to the hypothesis that the ability of epitope-specific CD8⁺ T cell responses to recognize the common escape mutations in the B27-GRAAICGKY epitope in some subjects may function to select for these rare and highly deleterious mutations. To better address this issue, we first determined the HLA-B27 binding capacity of the different epitope peptide variants. Indeed, a gradual effect of the different amino acid substitutions was observed (Table 2). Substitutions I₂₉₄₀T and I₂₉₄₀V did not show a substantial effect on HLA-B27 binding, consistent with the location of residue I₂₉₄₀ in the core region of the epitope (amino acid position 5), which likely is a T cell receptor contact site rather than an HLA-B27 binding anchor. The frequently observed R₂₉₃₇K substitution still showed some level of HLA-B27 binding, albeit at a strongly reduced level (IC₅₀ 1893nM compared to 31nM observed for the wild-type peptide). The fact that this variant still showed some degree of HLA-B27 binding is in accordance with the notion that lysine (K) has been found in a small portion of natural HLA-B27 ligands (13), and also with our previous finding that an arginine to lysine substitution in another HCV-specific HLA-B27 restricted CD8⁺ T cell epitope had no effect on HLA-B27 binding and only a minor effect on T cell recognition (9). Peptides with the combined R₂₉₃₇K/I₂₉₄₀V mutations or the R₂₉₃₇S mutation showed only borderline HLA-B27 binding capacity (IC₅₀ 4668 and 4815nM, respectively), while peptides with the R₂₉₃₇G substitution showed no HLA-B27 binding at all (IC₅₀ >75,000 nM). These results suggest that some of the epitope-specific escape mutations may still be able to be presented and cross-recognized by specific CD8⁺ T cells.

To determine whether variant-specific CD8⁺ T cell responses are capable of recognizing some of these escape mutations, we tested two B27-positive patients with an acute-resolving course of HCV genotype 1a infection and 12 B27-positive patients with a chronic HCV genotype 1a infection for responses against this epitope. We could establish peptide-specific CD8⁺ T cell lines from both patients with acute-resolving infection as well as from two out of the twelve patients with chronic infection. Of note, this low rate of detection in chronic HCV infection is similar to what has been observed for other dominant HCV-specific CD8⁺ T cell responses (14). Next, the cross-recognition of different peptide variants by the wild-type specific T cell line was tested. As shown in Fig. 5, one of the two patients with acute-resolving infection showed a broad cross-recognition of the different peptide variants including the common R₂₉₃₇K and I₂₉₄₀T mutations. Here, only the R₂₉₃₇G/I₂₉₄₀T double mutant led to a complete loss of cross-recognition. In the second acute-resolving patient a rather narrow pattern of cross-recognition was observed with only the R₂₉₃₇S, I₂₉₄₀V, and I₂₉₄₀T variant peptides showing a relevant level of cross-recognition. Finally, in both of the patients with chronic infection a similarly narrow level of cross-recognition was observed (Fig. 5). In sum, these experiments suggest that in some patients a broad degree of peptide variant cross-recognition may drive the selection of rare and highly deleterious mutations such as the R₂₉₃₇G/I₂₉₄₀T double variant, while in other patients, weak T cell responses with a narrow degree of peptide variant cross-recognition select for frequent and well tolerated escape mutations or even allow replication of the wild type sequence.

Discussion

HLA-B27 is associated with a high rate of spontaneous viral clearance in HCV genotype 1 infection (2, 8). We have previously suggested that the immunodominant HLA-B27 restricted HCV-specific CD8⁺ epitope NS5B₂₈₄₁₋₂₈₄₉ (ARMILMTHF) might have an important role in this protective effect (7, 9) due to the fact that viral escape from this epitope requires the evolution of several combined mutations within the epitope. In that epitope, since mutations at the main HLA-B27 binding anchors cannot occur due to viral fitness costs, mutations at T cell contact residues are required for viral escape (9). However, due to a broad cross-recognition of these escape mutations, such TCR escape mutations need to occur in clusters in order to efficiently escape from this dominant CD8⁺ T cell response.

Here, we studied viral evolution in a second B27-restricted HCV-specific CD8⁺ T cell epitope in NS5B (NS5B₂₉₃₆₋₃₉₄₄, GRAAICGKY). In this epitope region, viral sequence variations were strongly enriched in HLA-B27⁺ patients with chronic HCV genotype 1a infection as compared to HLA-B27 negative patients. Although cross-sectional data from chronically infected patient cannot formally prove viral evolution, these data strongly indicate that these mutations are indeed HLA-B27 driven viral escape mutations. Indeed, two different patterns of viral escape mutations were observed in patients chronically infected with HCV genotype 1a. In the majority of patients, viral escape was mediated by a conservative mutation either at the main HLA-B27 binding anchor at position 2 (arginine to lysine) or at a T cell receptor contact residue at position 5 (isoleucine to threonine). These mutations were also observed in a longitudinal sequence analysis of two HLA-B27⁺ patients with acute infection (supplementary table S2), further supporting the notion that the sequence variations observed in chronically infected patients indeed reflect viral escape mutations. Importantly, these frequent mutations had no relevant effect on viral replication as assessed in the subgenomic replicon model. In contrast, in a small subset of HLA-B27⁺ patients, non-conservative mutations occurred at the main HLA-B27 binding anchor at position 2, replacing the long, positively charged residue arginine by the short, uncharged residue glycine or serine. These mutations led to a nearly abolished viral replication capability. The molecular basis for this effect is readily found by structural analysis of genotype 1a NS5B: These mutations of R2937 (position 2) disrupt a network of charge interactions at the base of NS5B's beta-flap, preventing correct interactions between the thumb domain and the linker element. These interactions have been proposed to be involved in the rate-limiting early steps of RNA synthesis by NS5B (12). Indeed, modulation of the interactions between thumb and linker through mutation of R2937 was recently found to be involved in replicative properties of strain JFH1 NS5B (15). Accordingly, detrimental escape mutations R2937S or R2937G could only co-evolve in the genotype 1a context in concert with two additional, compensatory mutations located 61 and 55 amino acids upstream of the epitope. The former compensatory mutation E2875K is expected to restore charge complementarity at the base of the beta-flap, while the latter P2881L/Q would additively enhance interaction with the linker at its other binding site in the thumb. While these observations provide an attractive explanation for the need of compensatory mutations in the presence of the R2937S and R2937G mutation, it is important to point out that conserved RNA structures in this region may also provide further functional restrictions on the evolution of viral escape mutations, as has been reported for the 3' end of the RNA coding for NS5B (16). Evolution of certain amino acid mutations could further be complicated by the need to change two nucleotides from the consensus sequence. Importantly, mutation R2937S can be obtained by a single nucleotide change. The same is true for the R2937G mutation; however, the HLA-B27⁺ individual displaying this mutation had two nucleotide changes, while the HLA-B27 negative patient with the R2937G mutation had a single nucleotide change (supplementary table S3).

The finding that the two compensatory mutations E₂₈₇₅ and P₂₈₈₁ could restore the viral replication capacity from <5% to 70%-100% of wild-type level allows two important conclusions. First, it may help to explain why associations between certain protective HLA alleles, CD8⁺ T cell responses or viral escape mutations and HCV viral loads, at least in the chronic phase, are hard to identify. Indeed, the high replication rate as well as the high mutation rate of HCV might allow the selection of compensatory mutations and restore replication levels similar to wild-type even in the presence of mutations that are highly detrimental if occurring alone. In agreement with this finding, the B27⁺ patient who displayed the rare R2937G/I2940T mutation in concert with the compensatory mutations E2875K and P2881L mutation even had an extraordinary high viral load (supplementary table S1). Second, this finding points out that a modest viral fitness cost, e.g. a reduction of replication levels by 30–50% as observed in previous studies (9, 17), may still have a substantial effect *in vivo*. Indeed, this effect may be analogous to the benefit derived by maintaining HIV-positive patients on 3TC therapy despite the presence of the M184V 3TC-resistance mutation because of the negative impact of M184V on viral replication (11). This is further supported by the notion that mutations that were associated with a modest viral fitness cost in the *in vitro* replicon assay (e.g., E₂₈₇₅K, P₂₈₈₁Q, and I₂₉₄₀V) were observed very rarely (0.3%, 1.3%, and 0.3%, respectively) in B27-negative patients (Table 1). In contrast, mutations that had no effect on viral replication levels (or replicated even at >100% compared to wild-type in the *in vitro* system) occurred more frequently in B27-negative individuals (e.g., P₂₈₈₁L, R₂₉₃₇K, and I₂₉₄₀T), although they were detected significantly more frequently in B27-positive patients. These findings are in line with the observation in chimpanzees that escape mutations with a low impact on viral replication are fixed over time, while escape mutations with a more dramatic effect on viral replication are not sustained and also revert in an *in vitro* model system (18). It is tempting to speculate that the well replicating P₂₈₈₁L mutation may occur in a minor quasispecies in those patients that select for one of the rare, detrimental escape mutations, enabling sufficient replication until the additional E₂₈₇₅K mutations can evolve and to evade the CD8⁺ T cell response.

Some of the effects observed in the subgenomic HCV replicon system might be due to the specific sequence of the replicon used. Here, we used a genotype 1b (strain con1) based replicon with NS5B from genotype 1a (strain H77). In order to exclude that the observed effects were due to the genotype 1b proteins, we confirmed the observations made in this hybrid replicon in a replicon that only contained genotype 1a (strain H77) sequences. Importantly, similar effects of these mutations were observed in the genotype 1a replicon (supplementary figure S1). It is also noteworthy that previous studies have identified mutations that facilitate replication in the replicon system, while exhibiting a negative effect on viral replication e.g. in the chimpanzee model (19). Thus, it is important to interpret data obtained from the *in vitro* system in the context of data available *in vivo*. As described above, the viral fitness data observed for individual mutations *in vitro* corresponded well to their frequency observed in patients *in vivo*, indicating that they were not due to cell-culture specific phenomena.

With the majority of HLA-B27⁺ patients spontaneously clearing HCV genotype 1 infection (2, 8), these data may help to explain the protective effect of HLA-B27 in HCV infection. We have previously shown that a dominant response against the HLA-B27 restricted CD8⁺ T cell epitope NS5B₂₈₄₁₋₂₈₄₉ (ARMILMTHF) contributes to protection by HLA-B27, and could further demonstrate that a broad cross-recognition of viral escape mutations, as well as limitations of viral escape through fitness costs, are determinants of this protection (9). The requirement of clustered escape mutations in that epitope results in a complicated pathway of escape to evade the immune response. During acute infection, this difficulty in rapidly achieving effective escape may provide the epitope-specific CD8⁺ T cell response sufficient time to clear the virus before effective escape mutations can develop. In the present study,

we reveal an equally complicated pathway of viral escape in a second neighboring HLA-B27 restricted CD8⁺ T cell epitope that requires the evolution of a network of two compensatory mutations in at least a subset of patients. This requirement for complex escape pathways for two epitopes restricted by HLA-B27 would hamper the development of effective escape mutations to both of these responses, and thus prevent a major determinant of persistent infection (20–22).

Of note, mutations that compensate for viral fitness costs associated with viral escape mutations have not been described previously for HCV, but have now been observed in the HLA-B27 restricted epitope described here, in an HLA-B57 restricted epitope (Oniangue-Ndza et al., personal communication), as well as in an HLA-A3 restricted epitope (23). Since all three HLA alleles have been described to be associated with spontaneous HCV clearance (2, 24), the requirement for compensatory mutations due to the high viral fitness costs of specific escape mutations may be a common characteristic of protective HLA alleles in HCV infection. As noted earlier, a similar requirement for compensatory mutations has been made for the immunodominant HLA-B27-restricted KK10 epitope in HIV Gag (3–4), as well as for the HLA B57-restricted KF11 epitope in Gag (25), each of which are believed to contribute importantly to the immune control of HIV associated with each of these alleles.

In sum, our data suggest that CD8⁺ T cell responses targeting the HLA-B27 restricted epitope NS5B₂₉₃₆₋₂₉₄₄ (GRAAICGKY) are uniquely capable of driving the selection of rare and highly deleterious mutations in some HLA-B27-positive patients. These escape mutations require the evolution of a network of compensatory mutations located outside the epitope in order to restore viral replication. This complicated pathway of viral escape may significantly contribute to viral control in the acute phase of infection and thus contribute to protection by HLA-B27 observed in HCV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all participating patients, clinical collaborators, and collaborators at the Broad Institute of MIT and Harvard who conducted the full-genome virus sequencing (10). In addition, we thank Ralf Bartenschlager (University of Heidelberg) for providing plasmid I341PILuc_NS3_3ET as well as Huh7-Lunet cells, and Charles Rice (Rockefeller University) for providing plasmid H77 (H/FL).

Financial support: This project was funded in part with Federal funds from the National Institute of Allergy and Infectious Disease (NIAID) under grant R01-AI067926 (TMA) U19 AI082630 (TMA, GL), with funds from the Deutsche Forschungsgemeinschaft (Emmy Noether-Programm, NE 1567/1-1 to CNH; KU 2250/1-1 to TK; FOR1202 to VL), with funds from the Bundesministerium für Bildung und Forschung (BMBF 01EO0803 to CNH), with funds from the French National Agency for Research on AIDS and Viral Hepatitis (ANRS, grant AO 2010/CSS4 to SB), and with funds from Association pour la recherche sur le cancer (ARC, postdoctoral fellowship to CCS).

Abbreviations

AIDS	acquired immunodeficiency syndrome
CTL	cytotoxic T lymphocyte
DC	dendritic cell
HCV	hepatitis C virus
HIV	human immunodeficiency virus

HLA	human leukocyte antigen
NS	non-structural protein
PBMC	peripheral blood mononuclear cell
RdRp	RNA-dependent RNA polymerase

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	B27 NS5B 2936-2944							n patients	(%)		
	2936 G	2937 R	2938 A	2939 A	2940 I	2941 C	2942 G			2943 K	2944 Y
Consensus	G	R	A	A	I	C	G	K	Y	7/19	(36.8)
B27+	K	-	-	-	T	-	-	-	-	4/19	(21.1)
	-	-	-	-	-	-	-	-	-	4/19	(21.1)
	K	-	-	-	V	-	-	R	-	1/19	(5.3)
	S	-	-	-	-	-	-	-	-	1/19	(5.3)
	G	-	-	-	-	T	-	-	-	1/19	(5.3)
B27-	-	-	-	-	-	-	-	R	-	300/385	(77.9)
	K	-	-	-	-	-	-	-	-	36/385	(9.4)
	-	-	-	-	T	-	-	-	-	17/385	(4.4)
	-	-	-	-	-	-	-	-	-	15/385	(3.9)
	-	-	-	-	M	-	-	-	-	9/385	(2.3)
	K	-	-	-	-	-	-	R	-	3/385	(0.8)
	G	-	-	-	-	-	-	-	-	1/385	(0.3)
	-	-	-	-	V	-	-	-	-	1/385	(0.3)
	-	-	-	-	-	-	-	D	-	1/385	(0.3)
	K	-	-	-	-	T	-	-	-	1/385	(0.3)
-	-	-	-	-	-	-	T	-	1/385	(0.3)	

Fig. 1. HLA-B27 driven sequence variation in the HLA-B27 restricted epitope NS5B2936-2944
 The HCV genotype 1a consensus sequence, the autologous viral sequence in 19 HLA-B27+ patients with chronic HCV genotype 1a infection, and the autologous viral sequence in 385 HLA-B27 negative patients with HCV genotype 1a infection are shown. Positions with HLA-B27 associated sequence polymorphisms ('footprints', compare table 1) are shown with a grey background.

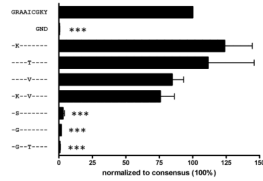


Fig. 2. Rare escape mutations at position two of the HLA-B27 restricted epitope NS5B₂₉₃₆₋₂₉₄₄ nearly abolish viral replication

A wild-type subgenomic replicon, a replication deficient variant (GND), and several variants containing escape mutations within the HLA-B27 restricted epitope NS5B₂₉₃₆₋₂₉₄₄ were tested for replication capacity. Replication of the wild-type was set 100%. Means and standard errors of three individual experiments are shown. P values comparing replication of mutants and wild-type were calculated by paired t test and are indicated as follows: * P<0.05, ** P<0.005, and *** P<0.0005.

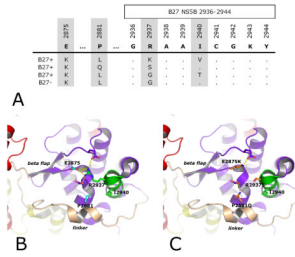


Fig. 3. Rare mutations R2937S, R2937G (with or without I2940T), and R2937K/I2940V within the HLAB27 restricted epitope NS5B2936-2944 only occur in concert with an equally rare cluster of two mutations 71 and 65 amino acids upstream of the epitope which restore a network of interactions in the NS5B thumb domain

A: Autologous viral sequences are shown for all patients that harbor the E2875K mutation (4/404 patients in the cohort; compare table 1). All four patients also display one of the rare mutations at position 2937 (R2937S, R2937G, or R2940K/I2940V). In addition, all four patients display a mutation at position 2881 (P2881L or P2881Q).

B: Close-up of the epitope region in the three-dimensional structure of genotype 1a NS5B. NS5B is displayed as ribbons and colored by domains (red: fingers; yellow: palm; purple: thumb). The epitope is shown in green with the sites of rare escape mutations R2937 and I2940 displayed as sticks, colored by atom type (blue: nitrogens; green: carbons) and labeled. The sites of compensatory mutations P2881 and E2875 are similarly displayed (red: oxygens; cyan: carbons) as well as two other acidic amino acids (purple: carbons) also in direct interaction (dotted yellow lines) with R2937. The thumb's 'beta-flap' is labeled in italics as well as the linker (in light brown) C-terminal to the thumb. **C:** Same view as in B, but with mutations R2937S, E2875K and P2881Q modeled into the structure (orange: carbons). E2875K compensates for the negative charge cluster at the base of the beta-flap that would be left by R2937S alone, restoring in part an orientation of the beta-flap that allows proper interaction with the linker. P2881Q completes the remodeling of the network of interactions between thumb and linker.

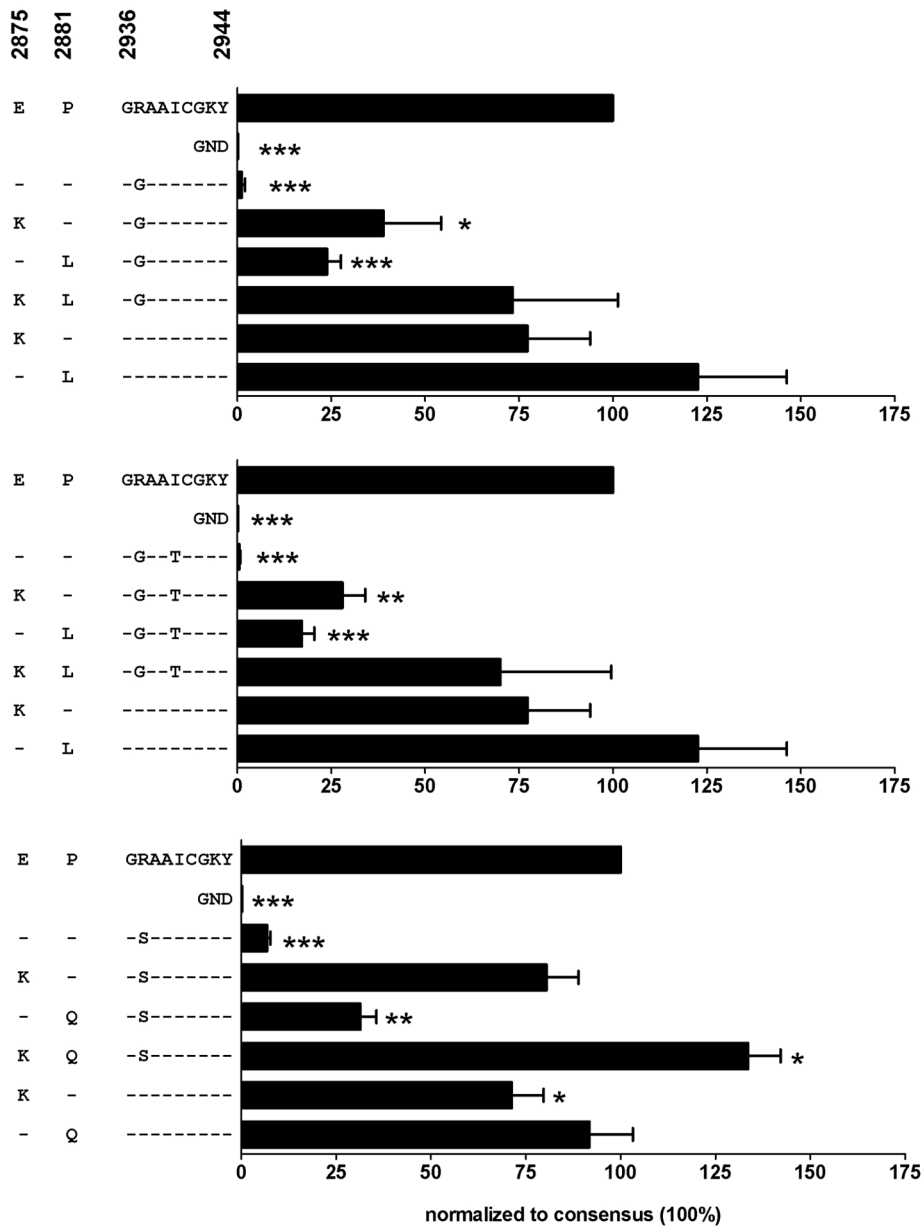


Fig. 4. Viral replication capacity of the rare mutants R2937S, R2937G, and R2937G/I2940T can be restored by mutations E2875K and P2881L/Q
 Replication levels of the wild-type subgenomic replicon, a replication deficient mutant (GND), mutants containing the rare escape mutations alone (R2937G, upper panel; R2937G/I2940T, middle panel; R2937S, lower panel), or in combination with mutations at position 2875 and/or 2881 were compared. Replication of the wild-type was set 100%. Means and standard errors of three individual experiments are shown. P values comparing replication of mutants and wild-type were calculated by paired t test and are indicated as follows: * P<0.05, ** P<0.005, and *** P<0.0005.

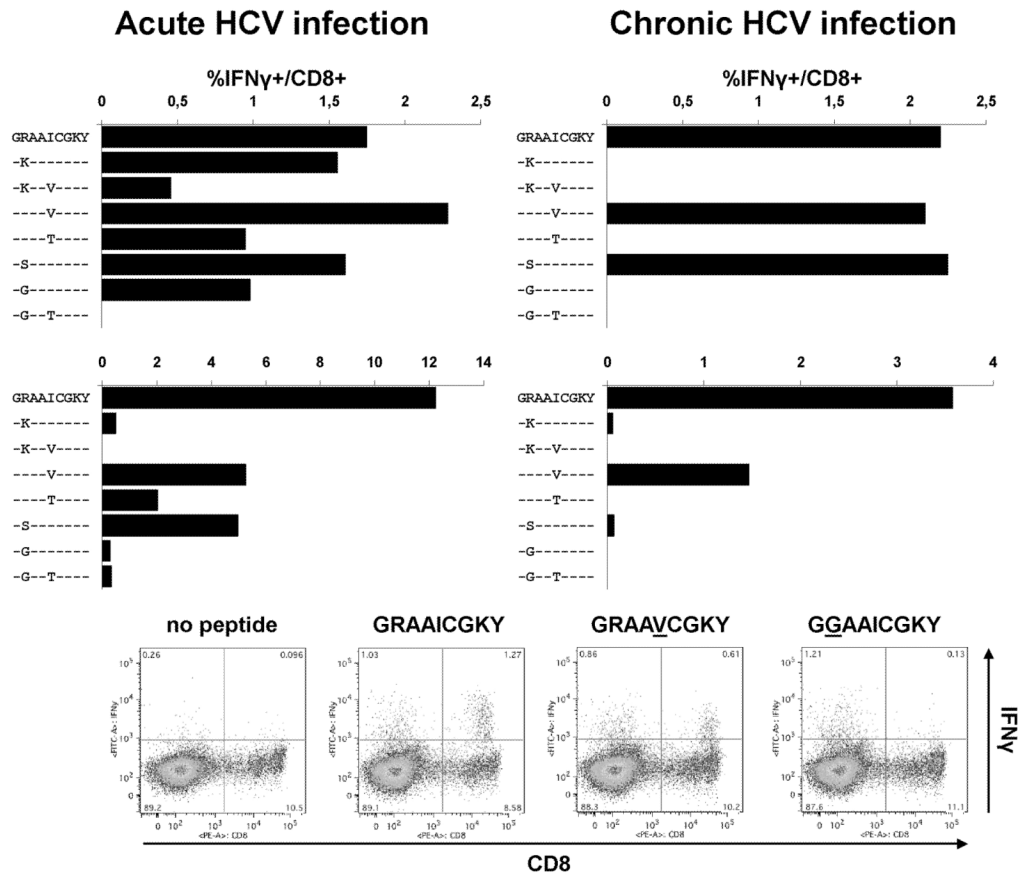


Fig. 5. NS5B₂₉₃₆₋₂₉₄₄ specific CD8+ T cell responses show varying degrees of variant cross-recognition in individual patients
 Epitope-specific T cell lines were derived from two HLA-B27+ patients with acute-resolving HCV genotype 1a infection (left panels) and from two HLA-B27+ patients with chronic HCV genotype 1a infection (right panels) and tested for interferon- γ production in response to wild-type peptide and variant peptides. Representative dot blots from the second patient with acute-resolving infection are shown below.

Table 1

Amino acid substitutions compared to consensus at Positions 2875, 2881, 2937, and 2940 in HLA-B27 positive and negative patients with chronic HCV genotype 1a infection.

Residue	Substitution	B27+ (n=19)	B27- (n=385)	P*
E2875	Q	0 (0.0%)	4 (1.0%)	1.0000
	K	3 (15.8%)	1 (0.3%)	0.0003
	any	3 (15.8%)	5 (1.3%)	0.0043
P2881	L	4 (21.1%)	24 (6.2%)	0.0347
	Q	1 (5.3%)	5 (1.3%)	0.2524
	S	0 (0.0%)	2 (0.5%)	1.0000
R2937	any	5 (26.3%)	31 (8.1%)	0.0183
	K	5 (26.3%)	21 (5.5%)	0.0047
	G	1 (5.3%)	1 (0.3%)	0.0920
I2940	S	1 (5.3%)	0 (0.0%)	0.0470
	any	7 (36.8%)	22 (5.7%)	0.0001
	T	5 (26.3%)	16 (4.2%)	0.0017
	M	0 (0.0%)	9 (2.3%)	1.0000
	V	1 (5.3%)	1 (0.3%)	0.0920
	any	6 (31.6%)	26 (6.8%)	0.0020

* P value: two-tailed Fisher's exact test comparing frequencies in B27+ versus HLA-B27- patients.

Table 2

Binding of epitope peptide variants to HLA-B*2705

Peptide	IC50 [nM]
GRAAICGKY	31
-K-----	1893
---T---	122
---V---	32
-K--V---	4668
-S-----	4815
-G-----	>75000
-G--T---	>75000