Received: 25 March 2018 | Accepted: 7 September 2018

DOI: 10.1111/jvh.13011

ORIGINAL ARTICLE

WILEY JOURNAL OF VIRAL HEPATITIS JVH

Differential escape of HCV from CD8$^+$ T cell selection pressure between China and Germany depends on the presenting HLA class I molecule

Youchen Xia[1,2] | Wen Pan[1] | Xiaoyu Ke[1,3] | Kathrin Skibbe[4] | Andreas Walker[4] | Daniel Hoffmann[5] | Yinping Lu[1] | Xuecheng Yang[1] | Xuemei Feng[1] | Qiaoxia Tong[1] | Jörg Timm[4] | Dongliang Yang[1]

[1]Department of Infectious Diseases, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

[2]Department of Gastroenterology and Hepatology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine (originally named "Shanghai First People's Hospital"), Shanghai, China

[3]Department of Emergency, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

[4]Institute of Virology, University Hospital Düsseldorf, Heinrich-Heine-University, Düsseldorf, Germany

[5]Bioinformatics and Computational Biophysics, Faculty of Biology, University of Duisburg-Essen, Essen, Germany

Correspondence
Dongliang Yang, Department of Infectious Diseases, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.
Email: dlyang@hust.edu.cn
and
Jörg Timm, Institut für Virologie, Heinrich-Heine-Universität Düsseldorf, Universitätsklinikum Düsseldorf, Düsseldorf, Germany.
Email: joerg.timm@med.uni-duesseldorf.de

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81461130019; International Science & Technology Cooperation Program of China, Grant/Award Number: 2011DFA31030; Deutsche Forschungsgemeinschaft (DFG TRR60 and TI323/4-1); WBE Liver Fibrosis Foundation, Grant/Award Number: CFHPC20171058

Summary

Adaptation of hepatitis C virus (HCV) to CD8$^+$ T cell selection pressure is well described; however, it is unclear if HCV differentially adapts in different populations. Here, we studied HLA class I-associated viral sequence polymorphisms in HCV 1b isolates in a Chinese population and compared viral substitution patterns between Chinese and German populations. We identified three HLA class I-restricted epitopes in HCV NS3 with statistical support for selection pressure and found evidence for differential escape pathways between isolates from China and Germany depending on the HLA class I molecule. The substitution patterns particularly differed in the epitope VTLTHPITK$_{1635\text{-}1643}$, which was presented by HLA-A*03 as well as HLA-A*11, two alleles with highly different frequencies in the two populations. In Germany, a substitution in position seven of the epitope was the most frequent substitution in the presence of HLA-A*03, functionally associated with immune escape and nearly absent in Chinese isolates. In contrast, the most frequent substitution in China was located at position two of the epitope and became the predominant consensus residue. Moreover, substitutions in position one of the epitope were significantly enriched in HLA-A*11-positive individuals in China and associated with different patterns of CD8$^+$ T cell reactivity. Our study confirms the differential escape pathways selected by HCV that depended on different HLA class I alleles in Chinese and German populations, indicating that HCV differentially adapts to distinct HLA class I alleles in these populations. This result has important implications for vaccine design against highly variable and globally distributed pathogens, which may require matching antigen sequences to geographic regions for T cell-based vaccine strategies.

Abbreviations: CHC, chronic hepatitis C; CTL, cytotoxic T lymphocyte; HCV, hepatitis C virus.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2018 The Authors. *Journal of Viral Hepatitis* Published by John Wiley & Sons Ltd

1 | INTRODUCTION

Globally, an estimated 71 million people have chronic hepatitis C virus (HCV) infection.[1] Although great success has been achieved in the antiviral therapy of chronic hepatitis C (CHC),[2] the lack of an effective prophylactic vaccine and the expensive cost for treatment make CHC a major health burden in developing countries. Several studies suggest that robust HCV-specific $CD8^+$ T cell responses directed at multiple epitopes are important for viral control.[3-6] Many vaccine candidates in current preclinical and clinical development therefore aim to efficiently activate T cells.[7-9] One of the major obstacles to vaccine development is the inherent sequence diversity of HCV.[10] Due to the lack of a proofreading function in the NS5B polymerase, HCV exists in the human host as a quasispecies with distinct variants.[11] The quasispecies nature of HCV enables rapid adaptation to changes in the replication environment. Accordingly, the rapid selection of variants by targeting cytotoxic T lymphocyte (CTL) epitopes is well documented and potentially associated with failure of immune control.[12-14]

CTL epitopes are presented by HLA class I molecules on the surface of an infected cell. Allelic variation at the HLA class I gene loci enables the binding and presentation of multiple different peptides derived from intracellular proteins. The enormous diversity of HLA class I molecules expressed in a population limits a pathogen's ability to evade immune pressure through adaptation to any individual host. Co-evolution of HLA class I-encoding genes with pathogens in a population was proposed as a driving force for this HLA diversity.[15,16] Of note, there are marked differences in the frequency of distinct HLA class I alleles within a single population but also between the frequencies of these alleles in distinct populations and different ethnic groups. Such differences between ethnic groups are the basis for the use of different T cell repertoires against viral infections.[17,18]

At the population level, reproducible escape in CD8 epitopes in subjects sharing the same restricting HLA class I allele drives associations between viral sequence polymorphisms and particular HLA alleles.[19-21] Host-to-host transmission of viral strains adapted to immune pressure has important consequences for vaccine development. Continuous accumulation of an escape mutation in a CD8 epitope in circulating isolates may eventually lead to deletion of the target antigen from the population. In support of this hypothesis, adaptation to the immune pressure of individual epitopes in the majority of circulating isolates was demonstrated in EBV and HIV.[22,23] In HIV, a fascinating observation suggested that expression of rare HLA alleles is a selection advantage and associated with lower viral load, presumably because circulating isolates may have adapted to frequent HLA alleles in a population.[24,25] In HCV, we similarly reported a relatively stable escape mutation in an immunodominant CD8 epitope in NS3 that replaced the immunogenic prototype epitope sequence in the majority of circulating isolates in Europe.[19] In a vaccine, such immune targets would likely be less efficient in providing protective immunity, as the relevant antigen is not present in most viral isolates.

The aim of this study was to address whether the adaptation of viral sequences to immune pressure is influenced by the HLA background of the host population. We sequenced HCV NS3 (genotype 1b) from circulating isolates in China and compared their sequences with the sequences of isolates from Germany. We utilized an algorithm to identify polymorphic sites that differ in isolates from these populations and compared viral substitution patterns between isolates from Chinese and German populations. By this approach, we found evidence for differential adaptation to the immune pressure of circulating HCV isolates in HLA-diverse populations.

2 | MATERIALS AND METHODS

2.1 | Patients

All Chinese patients were recruited at the outpatient department of infectious disease of the Union Hospital of the Huazhong University of Science and Technology in Wuhan, China. Only subjects with HCV genotype 1b infection and no evidence for acute hepatitis based on their history were included, and all patients were currently HCV-infected and untreated. All German patients were recruited at the hepatology outpatient clinics of the Essen University Hospital and in the ward for inpatient detoxification treatment of drug-addicted patients, as well as in the clinic for opiate substitution treatment at the Department of Addictive Behavior and Addiction Medicine, LVR Hospital Essen, Hospital of the University of Duisburg-Essen. The local ethics committee approved the study, and all patients gave written informed consent.

2.2 | PCR and sequencing of NS3

PCR and sequencing of the NS3 gene from 89 patient samples were performed in two overlapping fragments, essentially as previously described.[19] Viral RNA was extracted from 140 μL of serum utilizing the Qiagen vRNA extraction kit. Then, 10 μL of the eluted RNA served as a template for RT-PCR utilizing the Promega RT-PCR kit. Next, 2 μL of the PCR product served as a template for the first and second rounds of PCR with nested primers. The PCR products were directly sequenced with gene-specific primers, and the chromatograms were analysed and edited with program Codon Code Aligner. All sequences were submitted to GenBank (accession numbers: MG993225-MG993277, MG993280-MG993312 and MG993314-MG993319)

2.3 | Phylogenetic analyses

All nucleotide sequences from the Chinese and German samples were aligned with previously published sequences from a large HCV single-source outbreak ("East German anti-D cohort"[26]). Phylogenetic trees were constructed with the neighbour-joining method as implemented in Neighbor TreeMaker (available at https://

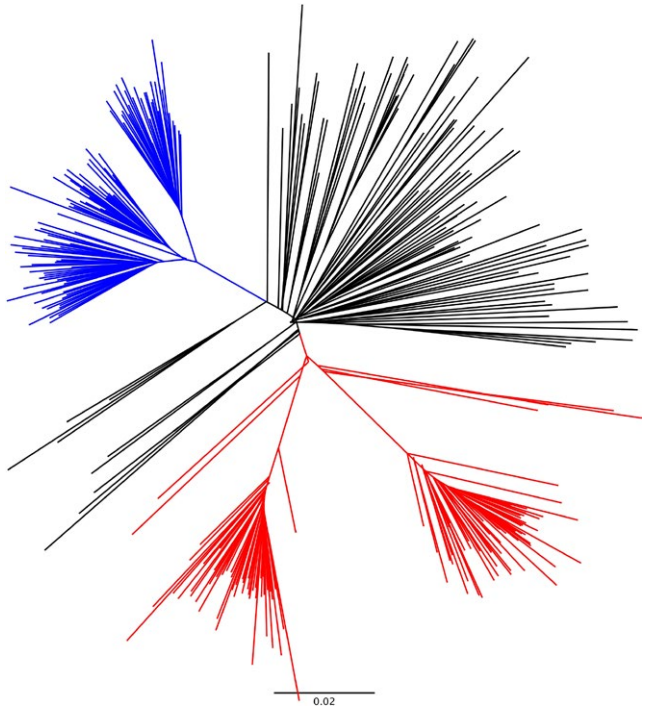

FIGURE 1 Phylogenetic tree of complete HCV NS3 sequences from China together with unrelated sequences and sequences from a single-source outbreak from Germany. Sequences from China (red) predominantly form two separate clades suggesting a common ancestor. Sequences from the anti-D cohort (blue) fall into three distinct clades. No clusters were formed within the German unrelated isolates (black)

hcv.lanl.gov/content/sequence/TREEMAKER/TreeMaker.html) using the General Time Reversible substitution model as suggested by FindModel (available at http://hcv.lanl.gov).

2.4 | HLA typing

Reference Chinese and German HLA frequencies were obtained from the HLA database (http://www.allelefrequencies.net). Only results from Bone Marrow Registry study and the Golden population standard were selected (China Jiangsu: sample size 3238, Germany pop 8: sample size 39 689). All the HLA-A alleles with frequencies higher than 0.1 and HLA-B alleles with frequencies higher than 0.05 were included.

HLA-A and HLA-B typing at the two-digit level were performed by using sequence-specific primer methodology (LABType methodology, both provided by One Lambda Inc., Canoga Park, CA, USA) at the Institute of Transfusion Medicine, Essen University Hospital, Essen, Germany.

2.5 | Analysis of associations between mutations and HLA class I alleles

The majority of consensus sequences were generated for Chinese and German isolates and served as references. Residues under selection pressure in the presence of individual HLA class I alleles were identified in a statistical approach utilizing the analysis tool SeqFeatR.[27]

2.6 | Analysis of the CD8$^+$ T cell response

Antigen-specific T cells were expanded from cryopreserved HLA-A*11- and A*03-positive PBMCs. Synthetic peptides were purchased from EMC, Tubingen, Germany. PBMCs were cultured in R10 medium (RPMI 1640 medium containing 10% foetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES buffer and 25 U/mL recombinant interleukin-2) and stimulated with different prototype peptides (1 μg/mL per peptide) and 0.1 μg/mL anti-CD28 and anti-CD49d. On day 7, medium containing IL-2 was added. On day 10, the cells were restimulated with the prototype or variant peptides in the presence of brefeldin A (100 ng/mL) for 4 hours, and the frequency of IFNγ-secreting CD8$^+$ T cells was analysed via flow cytometry. All samples were acquired using a FACS Canto (BD), and the data were analysed using FlowJo software (Tree Star Inc., Ashland, OR, USA)

2.7 | Statistical analysis

All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | Phylogenetic analysis revealed two distinct clades in Chinese isolates

The complete NS3 region was sequenced from 89 Chinese and 96 German patients. Phylogenetic analyses of isolates from China and Germany together with additional sequences from a large single-source outbreak were performed. Interestingly, the Chinese sequences fell into two phylogenetically distinct clusters (Figure 1), suggesting a founder effect with at least two GT1b origins in China. The genetic distance between isolates within these two Chinese clusters was similar to the distance between isolates of the "East German anti-D outbreak" that occurred in 1978/1979,[28] while no clear clusters were formed in the remaining German isolates. It has been reported that there are two main origins of HCV infections in China due to rapid transmission and spread of selected isolates in the 1960s-1990s.[29,30]

3.2 | Differential escape of HCV from CD8$^+$ T cell selection pressure between China and Germany

We compared the Chinese and German HLA-A and HLA-B allele frequencies in our cohort with data from the HLA database.[31] While only minor differences between the reported frequencies of common HLA class I alleles and the observed frequencies in our cohorts were present, there were substantial differences between the frequencies in China and Germany. The most frequent

TABLE 1 Substitutions in described CD8 T cell epitopes with statistical support for selection pressure

	HLA	*P* value	Described epitope	Described HLA
Substitution in China				
V1635I	A*11	0.001152	$VTLTHPITK_{1635-1643}$	A*03/11
K1378R	B*51	0.008989	$IPFYGKAI_{1371-1380}$	B*51
Q1598L	B*58	0.007896	$RAQAPPPSWDQ_{1596-1606}$	B*58
Substitution in German				
I1641V	A*03	0.003797	$VTLTHPITK_{1635-1643}$	A*03/11
I1373V	B*51	0.0704	$IPFYGKAI_{1371-1380}$	B*51

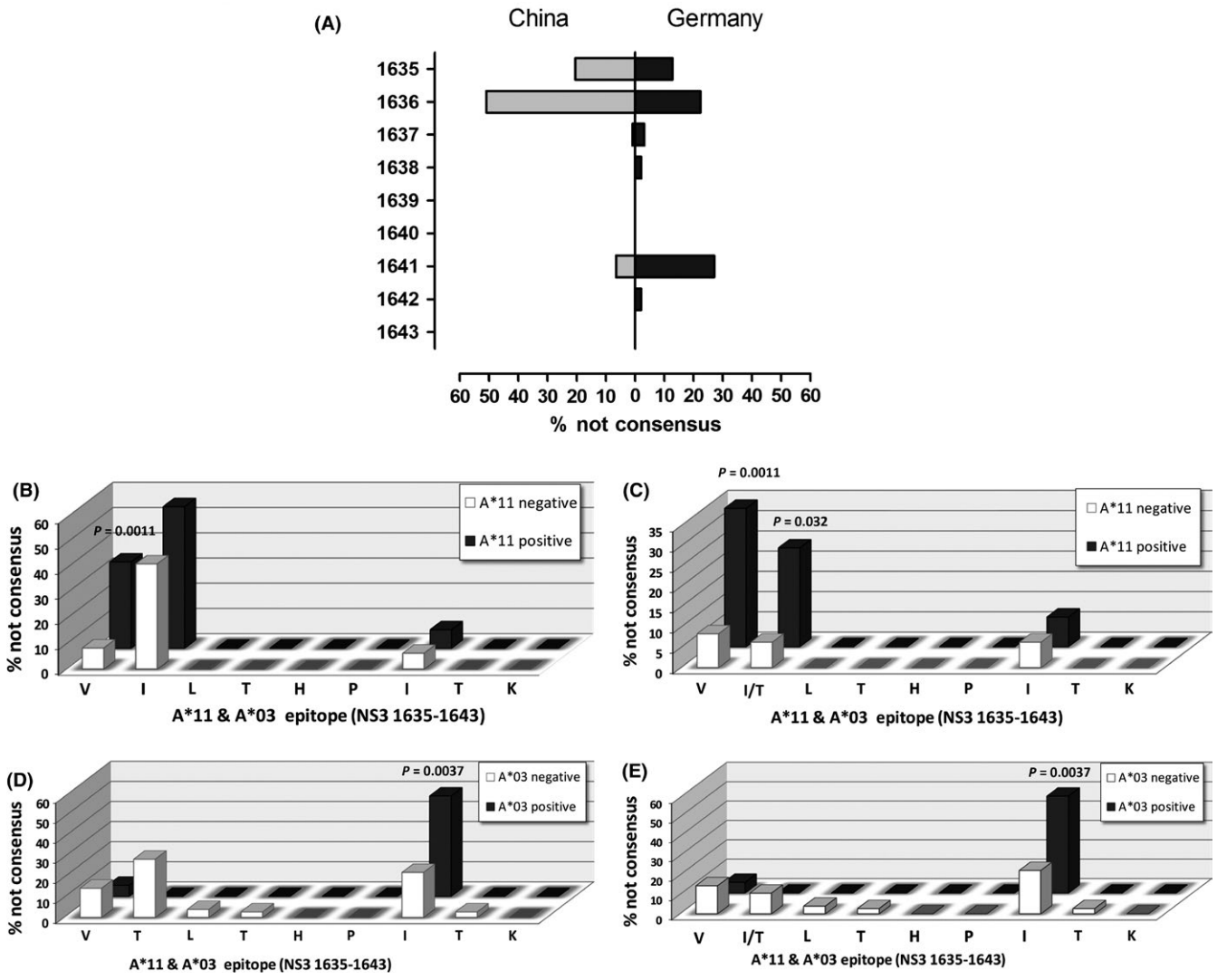

FIGURE 2 Frequency of HLA-A*11 and HLA-A*03 associated sequence polymorphisms in the $NS3_{1635-1643}$. A, The frequencies of variations from the consensus sequence in isolates from China (light grey) and Germany (dark grey) are shown. B and C, The frequency of variations from the consensus sequence in viral isolates from China from individuals carrying HLA-A*11 (dark grey) and individuals lacking HLA-A*11 (white) is shown for each position. In C, the frequency of variations from the amino acids isoleucine and threonine in position is shown. D and E, The frequency of variations from the consensus sequence in viral isolates from Germany from individuals carrying HLA-A*03 (dark grey) and individuals lacking HLA-A*03 (white) is shown for each position. In E, the frequency of variations from the amino acids isoleucine and threonine in position is shown. *P*-values were calculated with a Fisher's exact test

HLA allele in both populations is HLA-A*02. However, other frequent alleles at the HLA-A locus in China such as A*11 and A*24 are nearly absent from the German population. In turn, frequent alleles in Germany such as A*01 and A*03 are rare in China. Similar differences in HLA frequencies are also detected at the HLA-B locus (Table S1).

Given the evidence of HLA class I adaptation of HCV at the population level,[19,20,32] whether similar adaptation has occurred in Chinese isolates was addressed. We first analysed if viral sequence polymorphisms inside known CD8 T cell epitopes were statistically more frequent in the presence of the relevant HLA class I allele than in the absence of the relevant allele. The results are summarized in Table 1. In three previously described epitopes of NS3, there was statistical evidence for immune selection pressure on viral isolates from China by CD8 T cells. Notably, although the HLA-B*58-restricted epitope has been previously described in a Caucasian patient, there has been no evidence for immune selection.[33] The HLA-B*51-restricted epitope is also under selection pressure in German isolates, although a different substitution is selected in genotype 1b. One epitope region was particularly interesting because it was described to be presented by HLA-A*03 as well as HLA-A*11 ($VTLTHPITK_{1635\text{-}1643}$). In China, the substitution V1635I is selected in the presence of HLA-A*11, while in Germany, the substitution I1641V is selected in the presence of HLA-A*03.

The frequencies of sequence variants of this epitope are shown in more detail in Figure 2. There are three positions in the epitope showing a higher degree of sequence variation (Figure 2A). In position 1635, sequence variants are more frequent in China (20.4%) than in Germany (12.8%). The predominant substitution in this position in China (V1635I) is significantly enriched in the presence of HLA-A*11 ($P = 0.0011$; Figure 2B). Position 1636 is highly polymorphic in Chinese isolates, with isoleucine being the most prevalent amino acid in this position (49.0%) followed by threonine (38.0%), while other variants in this position (valine, asparagine, alanine, serine) were less frequent. Collectively, these less frequent variants were again significantly enriched in China in the presence of HLA-A*11 (Figure 2C), suggesting that rare variants are selected by HLA-A*11-restricted immune pressure. In German isolates, this position is more conserved with threonine being the most prevalent amino acid (76.6%), followed by isoleucine (14.9%). Interestingly, there was evidence that the consensus residue, threonine, was selected in the presence of HLA-A*03, as variations from threonine in position 1636 were only observed in the absence of HLA-A*03. There was no evidence for selection of rare variants in this position in the presence of HLA-A*03 (Figure 2E). In position 1641 sequence variants are more frequent in Germany (27.1%) than in China (6.5%). Here, the most frequent substitution (I1641V) was selected in the presence of HLA-A*03 ($P = 0.0037$; Figure 2D).

Taken together, we identified three HLA class I-restricted epitopes in HCV NS3 with statistical support for selection pressure and found evidence for distinct escape pathways between isolates from China and Germany depending on the presenting HLA class I molecule.

3.3 | Substitutions selected in the presence of HLA-A*11 and HLA-A*03 are associated with functional immune escape

The originally described HLA-A*03-restricted epitope in this region was a 9-mer ($VTLTHPITK_{1635\text{-}1643}$),[34,35] whereas the HLA-A*11-restricted epitope was originally described as an N-terminally truncated 8-mer ($TLTHPITK_{1636\text{-}1643}$).[36] Accordingly, the V1635I substitution selected in the presence of HLA-A*11 was one amino acid upstream of the originally described epitope. Therefore, the exact length of the epitope presented by HLA-A*11 was determined again by expansion of antigen-specific $CD8^+$ T cells from an HLA-A*11-positive patient with spontaneously resolved HCV infection with a 9-mer as well as with an 8-mer including either the German consensus residue (threonine) or the Chinese consensus residue (isoleucine). After 10 days of expansion, the cells were restimulated with the different variants and the frequency of IFNγ-producing $CD8^+$ T cells was determined by flow cytometry. Importantly, IFNγ-positive cells were only detectable when cells were expanded and restimulated with the 9-mer (Figure 3). In contrast, no IFNγ was produced upon restimulation of the same culture with the truncated 8-mer or upon expansion of PBMCs with an 8-mer, followed by restimulation with any variant. Collectively, the data indicate that in contrast to the originally described data, the 9-mer ($VTLTHPITK_{1635\text{-}1643}$) but not the 8-mer ($TLTHPITK_{1636\text{-}1643}$) is presented by HLA-A*11. Accordingly, the same peptide is presented by HLA-A*03 and HLA-A*11 with different residues being under selection pressure in the presence of both alleles.

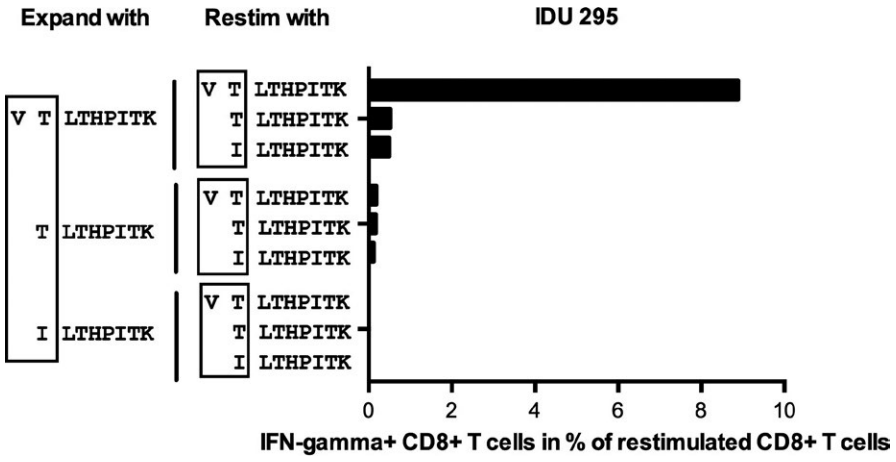

FIGURE 3 Characterization of the correct length of the epitope $NS3_{1635\text{-}1643}$ in the context of HLA-A*11. HLA-A*11 positive PBMCs were expanded for 10 days with the 9-mer peptide VTLTHPITK and truncated 8-mer variants and restimulated with different peptide variants as indicated. After intracellular cytokine staining, the frequency of IFN-gamma positive $CD8^+$ T cells was determined by flow cytometry and is shown in % of restimulated $CD8^+$ T cells

Next, the impact of sequence variants of the epitope on the CD8 T cell response was determined in the context of HLA-A*03 and HLA-A*11. PBMCs of three different HLA-A*03-positive HCV-exposed donors were used to expand antigen-specific T cells (Figure 4). Here, both the German consensus sequence (VTLTHPITK), which represents the originally described prototype epitope as well as the Chinese consensus sequence (VILTHPITK) were used for antigen-specific expansion. As expected, IFNγ-positive CD8$^+$ T cells were detected upon expansion and restimulation of PBMCs with the prototype epitope (VTLTHPITK). The I1641V substitution under selection pressure in the presence of HLA-A*03 functionally acts as an immune escape mutation, as the frequency of IFNγ-positive CD8$^+$ T cells was substantially reduced upon restimulation with this variant. Notably, the Chinese consensus did not activate antigen-specific CD8$^+$ T cells, suggesting that only the German consensus sequence is immunogenic in the context of HLA-A*03.

In the context of HLA-A*11, the impact of sequence variants of the epitope was more complex and differed among individuals. Two distinct patterns of CD8 T cell reactivity were distinguished. In the first pattern, CD8 T cells predominantly reacted with the German consensus sequence (VTLTHPITK) and the response to the Chinese sequence variant (VILTHPITK) was substantially weaker or nearly absent. In turn, when PBMCs were expanded in the presence of the

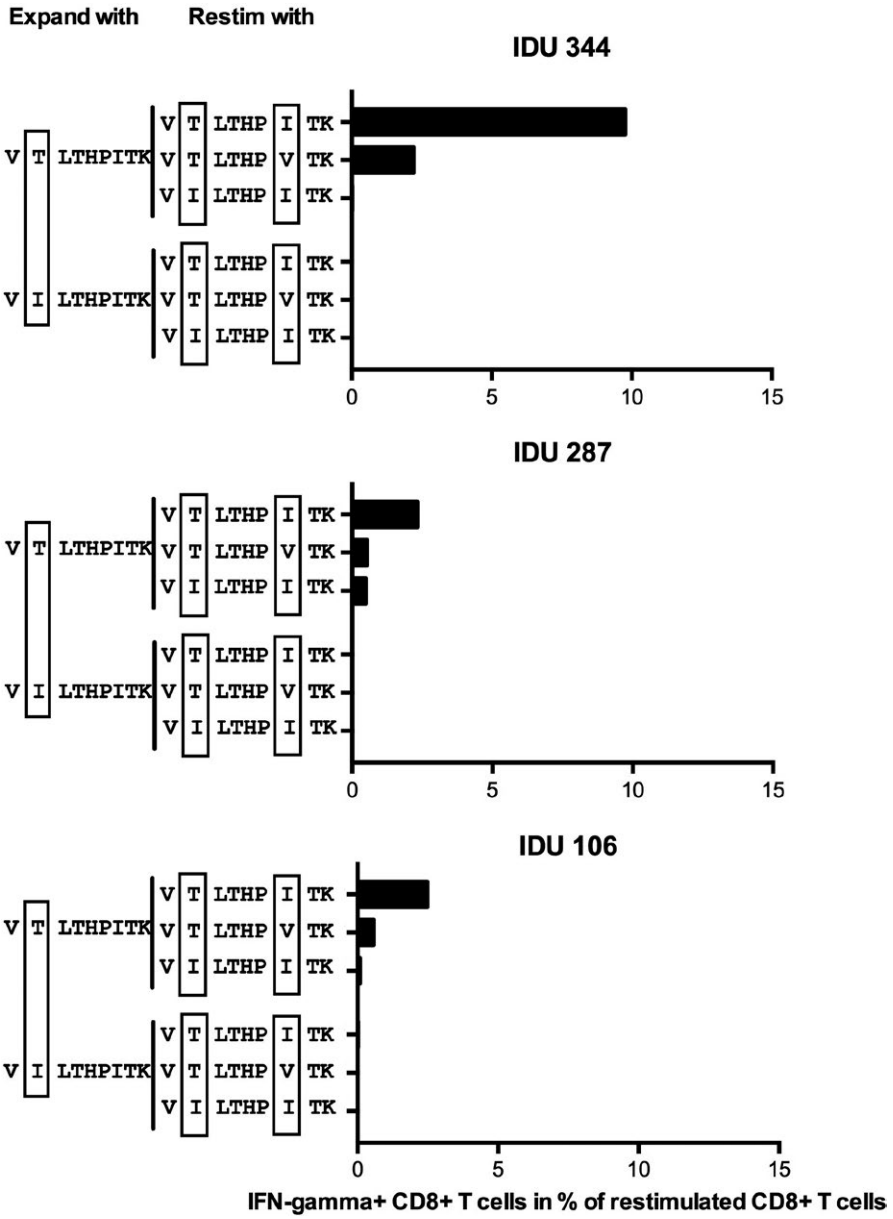

FIGURE 4 The substitution I1641V in the epitope $NS3_{1635\text{-}1643}$ is associated with functional immune escape in the context of HLA-A*03. HLA-A*03 positive PBMCs from three different donors were expanded with the peptide VTLTHPITK (German consensus) or VILTHPITK (Chinese consensus) for 10 days and then restimulated with different peptide variants as indicated. After intracellular cytokine staining, the frequency of IFN-gamma positive CD8$^+$ T cells was determined by flow cytometry and is shown in % of *restimulated CD8$^+$ T cells*

Chinese consensus no or substantially weaker CD8 T cell responses were detected, suggesting that the Chinese consensus sequence represents an escape variant. The I1641V substitution selected in the context of HLA-A*03 did not have a functional impact on the CD8 T cell response in the context of HLA-A*11. In contrast, the V1635I substitution within either the Chinese consensus sequence

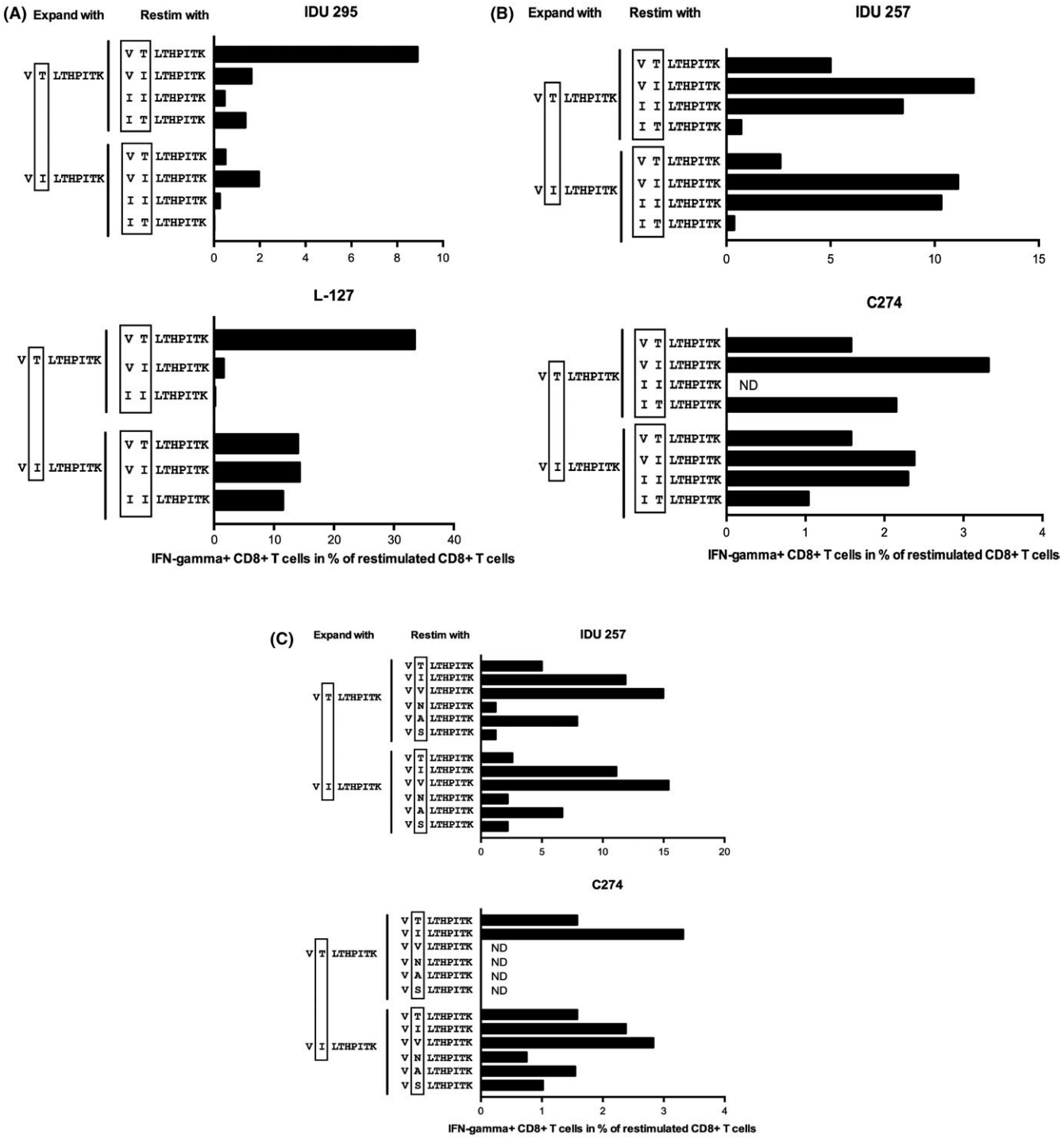

FIGURE 5 Two different patterns of immune escape from HLA-A*11-associated selection pressure. HLA-A*11 positive PBMCs from four different donors were expanded with the peptide VTLTHPITK (German consensus) or VILTHPITK (Chinese consensus) for 10 days and then restimulated with different peptide variants as indicated. After intracellular cytokine staining, the frequency of IFN-gamma positive $CD8^+$ T cells was determined by flow cytometry and is shown in % of *restimulated* $CD8^+$ *T cells*. Two distinct patterns of $CD8^+$ T cell reactivity were observed: A, In the first pattern, the response was predominantly directed against the German consensus (VTLTHPITK) and the Chinese consensus (VILTHPITK) as well as the V1635I variants were associated with lower $CD8^+$ T cell responses. B and C, In the second pattern, the response was predominantly directed against the Chinese consensus (VILTHPITK), here, the V1635I variant was not associated with a lower response (B). In this pattern, rare substitutions in position two of the epitope were associated with lower $CD8^+$ T cell responses (C)

(IILTHPITK) or the German consensus sequence (ITLTHPITK) both strongly impaired the $CD8^+$ T cell response, although the latter was unfortunately tested in only one individual (Figure 5A). Taking these results together, in the first pattern the CD8 T cell response is predominantly directed against the German consensus sequence (VTLTHPITK) and the Chinese consensus sequence and the V1635I substitution are both consistent with immune escape.

In the second pattern of CD8 T cell reactivity, the Chinese consensus sequence was more reactive than German consensus sequence. The IFNγ-positive CD8 T cells were more frequent against the Chinese consensus sequence (VILTHPITK) than against the German consensus sequence (VTLTHPITK) after the expansion of PBMCs in the presence of both epitope variants (Figure 5B). Here, the V1635I substitution within the Chinese consensus sequence (IILTHPITK) selected in the presence of HLA-A*11 did not have a functional impact on the CD8 T cell response. Importantly, other substitutions in position two of the epitope such as mutations to asparagine, alanine or serine impaired the immune response consistent with immune escape. Taking these results together, in the second pattern CD8 T cells are predominantly directed against the Chinese consensus sequence. In this pattern, the V1635I substitution does not impair the CD8 T cell response; however, the behaviour of some of the rare variants in position two of the epitope are consistent with immune escape.

4 | DISCUSSION

The ability of HCV to adapt to immune pressure by selection of mutations conferring resistance to components of the adaptive immune system within one infected individual is well documented.[10,12,14] Previous studies suggested that cellular immune selection pressure can be reproducible at the population level, resulting in "HLA class I footprints" in sequences of circulating isolates.[19,21,37] Here, we extend this analysis to sequences from different geographic regions and with host populations with HLA class I backgrounds.

One epitope region was particularly interesting because it was presented by two different HLA class I molecules, one that is frequent in Germany (HLA-A*03) but rare in China and one that is highly prevalent in China (HLA-A*11) but rare in Germany. In the context of HLA-A*11, the epitope was originally described as an 8-mer[36]; however, in our hands, no CD8 T cell response was detected against the 8-mer. In contrast, the N-terminally elongated 9-mer was targeted by CD8 T cells. Notably, the most frequent consensus residue in position 1636 in the epitope differed between circulating isolates from China and Germany. We found immunological support for immune-driven accumulation of the consensus residue isoleucine as an HLA-A*11 escape mutation in HLA-A*11-positive individuals in China. Moreover, at the sequence level, different positions were under selection pressure in Germany and in China. In China, substitutions at position 1635 were selected in the presence of HLA-A*11, whereas in Germany, substitutions at position 1641 were selected. Distinct selection pressure on the same epitope region that depended on the presenting HLA class I allele has been described in HIV[38]; however, this observation has not been linked to differences in populations. The data presented here are consistent with differential escape pathways by HCV in different populations.

Although the selected I1641V substitution was clearly associated with CD8 T cell immune escape in the context of HLA-A*03, the impact of sequence variation in the epitope region was more complex in the context of HLA-A*11. Two distinct patterns of CD8 T cell cross-reactivity were observed when the epitope was presented by HLA-A*11. In the first pattern, the German consensus sequence of the epitope was strongly reactive, but the Chinese consensus sequence carrying the T1636I substitution was not, consistent with immune escape of the Chinese consensus sequence. In contrast, the second pattern of CD8 T cell reactivity was characterized by a maximum response against the Chinese consensus sequence. In these patients, rare substitutions in position 1636 (to asparagine, alanine or serine) were functionally associated with immune escape. Notably, at the sequence level, there was statistical evidence that these rare substitutions were indeed predominantly selected in the presence of HLA-A*11 (Figure 2C). The reasons for the different cross-reactivity patterns of CD8 T cells in the context of HLA-A*11 are unclear. Individual differences between the TCR repertoires recruited for the immune response against this epitope may have contributed to the effect. Distinct TCR repertoires may be the consequence of different abundance of suitable TCRs in the naïve repertoire prior to T cell priming.[39] However, the original viral sequence at the time of transmission may also play a role in the cross-reactivity patterns. In a previous study, we analysed the consequences of sequence variation in an immunodominant HLA-A*02-restricted epitope on the cross-reactivity of in vitro primed CD8 T cells.[26] These data revealed that different epitope variants were associated with distinct and reproducible cross-reactivity patterns. In the context of HLA-A*11, similar sequence differences at the time of priming (eg, the T1636I sequence polymorphism) may have contributed to the different cross-reactivity patterns of CD8 T cells.

In Germany, there was even evidence for selection of threonine in position 1636 in the context of HLA-A*03, although the Chinese consensus sequence carrying the isoleucine in this position was consistent with immune escape in the context of HLA-A*03. This observation is more difficult to explain because the results of the immunological assays would argue for the preferential selection of the T1636I substitution. Other factors may prevent selection of this residue. It is possible that an overlapping epitope presented by a different HLA class allele alters the direction of selection pressure. Opposing selection forces on the same residue that depend on the presenting HLA class I allele have been described in HIV and HCV.[40] Negative selection pressure on isoleucine in position 1636 due to fitness constraints seems less likely, given that the accumulation of this substitution has occurred in China. However, it is possible that the genome plasticity depends on the larger sequence context and that the T1636I substitution is less fit in the context of GT1b isolates from Germany and more fit in the context of GT1b isolates from China. In line with this reasoning, clade-specific differences in

the functional constraints associated with different escape pathways has been described for HIV.[41] In the phylogenetic tree of the HCV sequences, the Chinese isolates form two separate clusters that are possibly associated with different sequence constraints. Unfortunately, the relevance of individual substitutions for viral fitness in different genetic GT1b backgrounds is difficult to address in cell culture experiments, as only few GT1b isolates support replication in vitro.[42]

The phylogenetic tree confirms previous observations suggesting the rapid spread of individual GT1b isolates between 1960 and 1990 in China.[29,30] In line with this possibility, the genetic distance between Chinese isolates is similar to the genetic distance between isolates from a large HCV GT1b outbreak that occurred in the former German Democratic Republic between 1977/78.[28] Accordingly, founder effects in China may play a role for sequence differences between isolates from Germany and China.[43] It is possible that the transmission and rapid spread of a few isolates carrying the T1636I substitution resulted in different consensus sequences in Germany and China. Nevertheless, the impact of the substitution on the immune response in the context of the highly prevalent allele HLA-A*11 in China may have promoted the transmission of this viral variant.

It was previously reported in a large HCV genotype 1b outbreak in Ireland that HLA-A*03 is associated with spontaneous clearance of HCV infection.[44] The protective effect was linked to targeting of a dominant epitope located in NS3 (TVYHGAGTK) and fitness costs associated with individual escape mutations requiring additional compensatory mutations.[45,46] Notably, the protective effect was exclusively observed in the Irish outbreak and not in a similar HCV genotype 1b outbreak in East Germany, possibly because the immunodominant target in NS3 differed between the sources of these outbreaks.[40] There are no data available suggesting that HLA-A*11 has a differential impact on the outcome of HCV infection; however, no such studies have been performed in Asian cohorts. Reproducible selection pressure on different residues within the epitope and a complex escape pathway, as observed here for HLA-A*11, would be consistent with a beneficial effect of HLA-A*11 for disease outcome.

Collectively, our data are consistent with differential escape pathways selected by HCV in the context of different HLA class I alleles. This result has important implications for vaccine design against highly variable and globally distributed pathogens. Our results suggest that pathogens such as HCV differentially adapt in HLA-diverse populations, which may require matching antigen sequences to geographic regions for T cell-based vaccine strategies.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (81461130019), the International Science & Technology Cooperation Program of China (2011DFA31030), Transregio TRR60 (Deutsche Forschungsgemeinschaft) and the WBE Liver Fibrosis Foundation (Grant No. CFHPC20171058).

CONFLICT OF INTEREST

The authors declare there is no conflict of interest regarding the publication of this paper.

ORCID

Youchen Xia http://orcid.org/0000-0001-9241-884X
Jörg Timm http://orcid.org/0000-0001-7799-3045
Dongliang Yang http://orcid.org/0000-0001-5387-2660

REFERENCES

1. WHO. Hepatitis C. 2017. http://www.who.int/mediacentre/factsheets/fs164/en/. Accessed January 25, 2018.
2. Asselah T, Boyer N, Saadoun D, Martinotpeignoux M, Marcellin P. Direct-acting antivirals for the treatment of hepatitis C virus infection: optimizing current IFN-free treatment and future perspectives. *Liver Int*. 2016;36(Suppl. 1):47-57.
3. Thimme R, Bukh J, Spangenberg HC, et al. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci*. 2002;99:15661-15668.
4. Thimme R, Oldach D, Chang K-M, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med*. 2001;194:1395-1406.
5. Grüner NH, Gerlach TJ, Jung M-C, et al. Association of hepatitis C virus—specific CD8 + T cells with viral clearance in acute hepatitis C. *J Infect Dis*. 2000;181:1528-1536.
6. Lechner F, Wong DK, Dunbar PR, et al. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med*. 2000;191:1499-1512.
7. Haller AA, Lauer GM, King TH, et al. Whole recombinant yeast-based immunotherapy induces potent T cell responses targeting HCV NS3 and Core proteins. *Vaccine*. 2007;25:1452-1463.
8. Klade CS, Wedemeyer H, Berg T, et al. Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. *Gastroenterology*. 2008;134:1385-1395. e1381.
9. Zubkova I, Choi YH, Chang E, et al. T-cell vaccines that elicit effective immune responses against HCV in chimpanzees may create greater immune pressure for viral mutation. *Vaccine*. 2009;27:2594-2602.
10. Timm J, Walker CM. Mutational escape of CD8 + T cell epitopes: implications for prevention and therapy of persistent hepatitis virus infections. *Med Microbiol Immunol*. 2015;204:29-38.
11. Scheel TK, Rice CM. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nat Med*. 2013;19:837-849.
12. Cox AL, Mosbruger T, Mao Q, et al. Cellular immune selection with hepatitis C virus persistence in humans. *J Exp Med*. 2005;201:1741-1752.
13. Tester I, Smyk-Pearson S, Wang P, et al. Immune evasion versus recovery after acute hepatitis C virus infection from a shared source. *J Exp Med*. 2005;201:1725-1731.
14. Timm J, Lauer GM, Kavanagh DG, et al. CD8 epitope escape and reversion in acute HCV infection. *J Exp Med*. 2004;200:1593-1604.
15. Kiepiela P, Leslie AJ, Honeyborne I, et al. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature*. 2004;432:769-775.
16. Meyer D, Thomson G. How selection shapes variation of the human major histocompatibility complex: a review. *Ann Hum Genet*. 2001;65:1-26.

17. Sugimoto K, Stadanlick J, Ikeda F, et al. Influence of ethnicity in the outcome of hepatitis C virus infection and cellular immune response. *Hepatology*. 2003;37:590-599.
18. Tan AT, Loggi E, Boni C, et al. Host ethnicity and virus genotype shape the hepatitis B virus-specific T-cell repertoire. *J Virol*. 2008;82:10986-10997.
19. Neumann-Haefelin C, Frick DN, Wang JJ, et al. Analysis of the evolutionary forces in an immunodominant CD8 epitope in hepatitis C virus at a population level. *J Virol*. 2008;82:3438-3451.
20. Timm J, Li B, Daniels MG, et al. Human leukocyte antigen-associated sequence polymorphisms in hepatitis C virus reveal reproducible immune responses and constraints on viral evolution. *Hepatology*. 2007;46:339-349.
21. Gaudieri S, Rauch A, Park LP, et al. Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection. *J Virol*. 2006;80:11094-11104.
22. de Campos-Lima P-O, Gavioli R, Zhang Q-J, et al. HLA-A11 epitope loss isolates of Epstein-Barr virus from a highly A11 + population. *Science*. 1993;260:98-100.
23. Leslie A, Kavanagh D, Honeyborne I, et al. Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. *J Exp Med*. 2005;201:891-902.
24. Trachtenberg E, Korber B, Sollars C, et al. Advantage of rare HLA supertype in HIV disease progression. *Nat Med*. 2003;9:928-935.
25. Carlson JM, Du VY, Pfeifer N, et al. Impact of pre-adapted HIV transmission. *Nat Med*. 2016;22:606-613.
26. Ziegler S, Skibbe K, Walker A, et al. Impact of sequence variation in a dominant HLA-A*02-restricted epitope in HCV on priming and cross-reactivity of CD8 + T cells. *J Virol*. 2014;88:11080-11090.
27. Budeus B, Timm J, Hoffmann D. SeqFeatR for the discovery of feature-sequence associations. *PLoS ONE*. 2016;11:e0146409.
28. Ruhl M, Knuschke T, Schewior K, et al. CD8 + T-cell response promotes evolution of hepatitis C virus nonstructural proteins. *Gastroenterology*. 2011;140:2064-2073.
29. Lu L, Nakano T, He Y, Fu Y, Hagedorn CH, Robertson BH. Hepatitis C virus genotype distribution in China: predominance of closely related subtype 1b isolates and existence of new genotype 6 variants. *J Med Virol*. 2005;75:538-549.
30. Nakano T, Lu L, He Y, Fu Y, Robertson BH, Pybus OG. Population genetic history of hepatitis C virus 1b infection in China. *J Gen Virol*. 2006;87:73-82.
31. González-Galarza FF, Takeshita LY, Santos EJ, et al. Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res*. 2015;43:D784-D788.
32. Ruhl M, Chhatwal P, Strathmann H, et al. Escape from a dominant HLA-B*15-restricted CD8 + T cell response against hepatitis C virus requires compensatory mutations outside the epitope. *J Virol*. 2012;86:991-1000.
33. Neumann-Haefelin C, Timm J, Spangenberg HC, et al. Virological and immunological determinants of intrahepatic virus-specific CD8 + T-cell failure in chronic hepatitis C virus infection. *Hepatology*. 2008;47:1824-1836.
34. Giugliano S, Oezkan F, Bedrejowski M, et al. Degree of cross-genotype reactivity of hepatitis C virus-specific CD8+ T cells directed against NS3. *Hepatology*. 2009;50:707-716.
35. Mizukoshi E, Nascimbeni M, Blaustein JB, et al. Molecular and immunological significance of chimpanzee major histocompatibility complex haplotypes for hepatitis C virus immune response and vaccination studies. *J Virol*. 2002;76:6093-6103.
36. Cucchiarini M, Kammer AR, Grabscheid B, et al. Vigorous peripheral blood cytotoxic T cell response during the acute phase of hepatitis C virus infection. *Cell Immunol*. 2000;203:111-123.
37. Ray SC, Fanning L, Wang X-H, Netski DM, Kenny-Walsh E, Thomas DL. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. *J Exp Med*. 2005;201:1753-1759.
38. Leslie A, Price DA, Mkhize P, et al. Differential selection pressure exerted on HIV by CTL targeting identical epitopes but restricted by distinct HLA alleles from the same HLA supertype. *J Immunol*. 2006;177:4699-4708.
39. Chen H, Ndhlovu ZM, Liu D, et al. TCR clonotypes modulate the protective effect of HLA class I molecules in HIV-1 infection. *Nat Immunol*. 2012;13:691-700.
40. Ziegler S, Ruhl M, Tenckhoff H, et al. Susceptibility to chronic hepatitis C virus infection is influenced by sequence differences in immunodominant CD8 + T cell epitopes. *J Hepatol*. 2013;58:24-30.
41. Payne RP, Branch S, Kløverpris H, et al. Differential escape patterns within the dominant HLA-B*57:03-restricted HIV Gag epitope reflect distinct clade-specific functional constraints. *J Virol*. 2014;88:4668-4678.
42. Vieyres G, Pietschmann T. Entry and replication of recombinant hepatitis C viruses in cell culture. *Methods*. 2013;59:233-248.
43. Bhattacharya T, Daniels M, Heckerman D, et al. Founder effects in the assessment of HIV polymorphisms and HLA Allele Associations. *Science*. 2007;315:1583-1586.
44. McKiernan SM, Hagan R, Curry M, et al. Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *Hepatology*. 2004;40:108-114.
45. Karen F, Danijela P, Narayan R, et al. Molecular footprints reveal the impact of the protective HLA-A*03 allele in hepatitis C virus infection. *Gut*. 2011;60:1563-1571.
46. Merani S, Petrovic D, James I, et al. Effect of immune pressure on hepatitis C virus evolution: insights from a single-source outbreak. *Hepatology*. 2011;53:396-405.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Xia Y, Pan W, Ke X, et al. Differential escape of HCV from CD8$^+$ T cell selection pressure between China and Germany depends on the presenting HLA class I molecule. *J Viral Hepat*. 2019;26:73–82. https://doi.org/10.1111/jvh.13011