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Article

Infectious disease

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# Hepatitis C virus mutation affects proteasomal epitope processing

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**The high incidence of hepatitis C virus (HCV) persistence raises the question of how HCV interferes with host immune responses. Studying a single-source HCV outbreak, we identified an HCV mutation that impaired correct carboxyterminal cleavage of an immunodominant HLA-A2–restricted CD8 cell epitope that is frequently recognized by recovered patients. The mutation, a conservative HCV nonstructural protein 3 (NS3) tyrosine to phenylalanine substitution, was absent in 54 clones of the infectious source, but present in 15/21 (71%) HLA-A2–positive and in 11/24 (46%) HLA-A2–negative patients with chronic hepatitis C. In order to analyze whether the mutation affected the processing of the HLA-A2–restricted CD8 cell epitope, mutant and wild-type NS3 polypeptides were digested *in vitro* with 20S constitutive proteasomes and with immunoproteasomes. The presence of the mutation resulted in impaired carboxyterminal cleavage of the epitope. In order to analyze whether impaired epitope processing affected T cell priming *in vivo*, HLA-A2–transgenic mice were infected with vaccinia viruses encoding either wild-type or mutant HCV NS3. The mutant induced fewer epitope-specific, IFN- $\gamma$ –producing and fewer tetramer<sup>+</sup> cells than the wild type. These data demonstrate how a conservative mutation in the flanking region of an HCV epitope impairs the induction of epitope-specific CD8<sup>+</sup> T cells and reveal a mechanism that may contribute to viral sequence evolution in infected patients.**

## Introduction

Hepatitis C virus (HCV) is a 9.6 kb positive-stranded RNA virus of the flavivirus family and the leading cause of chronic hepatitis worldwide. Whereas recovery from acute HCV infection has been associated with multispecific T cell responses that protect upon reexposure to the virus (1, 2), these responses are either not induced or not maintained in the large number of patients who develop chronic infection (3–5).

We have therefore asked whether HCV interferes with the induction of antigen-specific T cells. Induction of CD8<sup>+</sup> T cells depends on the generation of MHC class I ligands by the proteasome, the major cytosolic proteinase. The proteasome cleaves short peptides from longer polypeptide precursors that are then translocated into the endoplasmic reticulum and bind to newly synthesized MHC class I molecules (6). 26S proteasomes contain a 20S catalytic core, arranged as 2 heptameric outer rings with 7  $\alpha$ -subunits each and 2 heptameric inner rings with 7  $\beta$ -subunits each. Proteasome activity is closely regulated by cytokines that are produced in viral infections (7–12). In response to IFN- $\gamma$ , for example, the constitutive catalytic subunits  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 are replaced by low molecular weight protein 2 (LMP2) (i $\beta$ 1), LMP7 (i $\beta$ 5), and multicatalytic endopeptidase complex-like-1 (MECL-1) (i $\beta$ 2) to form immunoproteasomes with altered cleavage properties (13).

HCV circulates in an abundant number of quasispecies because of its high replication rate (14) and its lack of polymerase proof-

reading capacity. Individual HCV sequences have been described as abolishing recognition by T cell receptors (TCRs) and antibodies, thus interfering with the effector arms of the cellular (2, 15, 16) and humoral immune responses (17). In contrast, the possibility that HCV mutations affect the induction of T cell responses has not been investigated. Indirect evidence for this hypothesis stems from descriptive reports that HCV isolates from persistently infected patients often encode less immunogenic sequences than prototype peptides used for *in vitro* analysis (18, 19). Whether the decreased immunogenicity results from viral mutations or from infection with less immunogenic strains has not been analyzed because the sequence of the infecting virus is not known in most human studies and also because the route of infection and inoculum size differ among the studied individuals.

Having studied a cohort of patients accidentally infected by a single-source HCV with known sequence, we here demonstrate that an HCV mutation located in the flanking region of a frequently recognized HCV epitope (4, 20–25) impairs the induction of HCV-specific CD8<sup>+</sup> T cells by affecting the sophisticated proteasomal antigen-processing machinery.

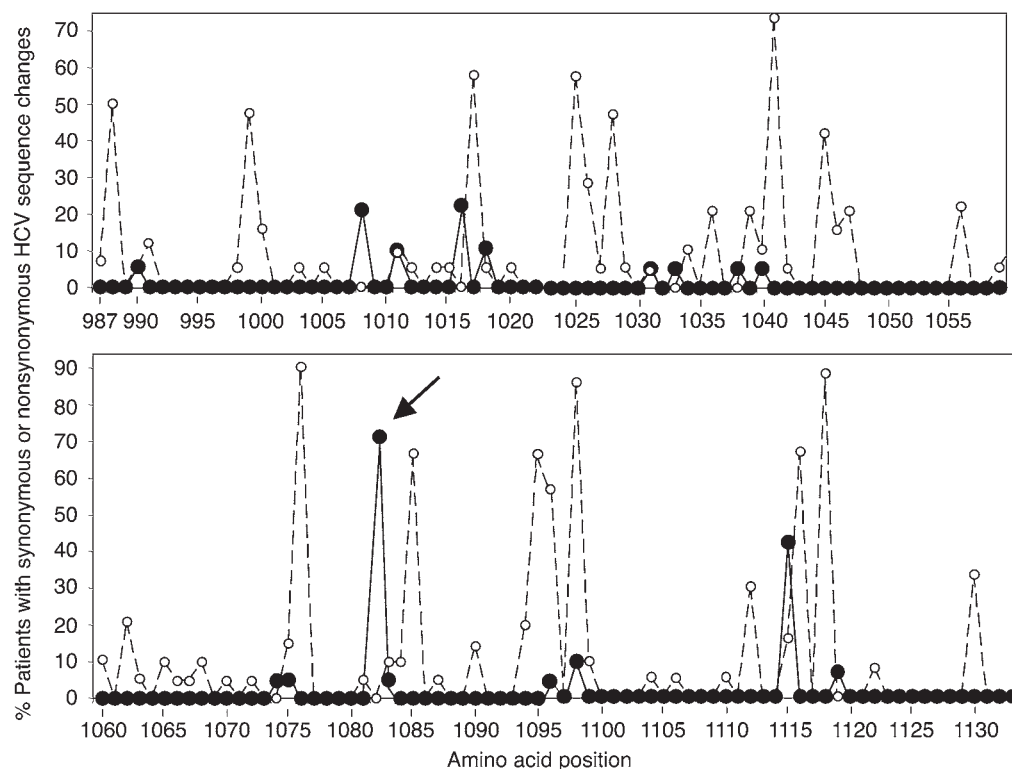
## Results

*The tyrosine/phenylalanine mutation at residue HCV nonstructural protein 3<sub>1082</sub> is common in patients who develop persistent infection after a single-source outbreak of HCV.* In order to study viral mutations during the natural course of HCV infection in humans, we analyzed a cohort of patients that had accidentally been infected with HCV in 1978/1979 during a single-source outbreak due to a contaminated anti-D immunoglobulin (26). Because the precise time of infection as well as the genotype and the sequence of the original infectious virus were known and identical for all patients, this cohort was

**Nonstandard abbreviations used:** hepatitis C virus (HCV); nonstructural protein 3 (NS3); phenylalanine (F); tyrosine (Y).

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**Figure 1**

Prevalence of synonymous (open circles) and non-synonymous (filled circles) HCV mutations within the HCV NS3<sub>987-1133</sub> sequence isolated from HLA-A2 positive, persistently infected HCV patients 18 years after a single-source outbreak of hepatitis C. The arrow indicates the amino acid position with the highest rate of non-synonymous to synonymous mutations.

suitable for studying candidate mechanisms of viral persistence. As previously described for this and other cohorts, the strength of the cellular immune response correlated with the outcome of infection, and HCV nonstructural protein 3 (NS3) peptides were among the most frequently recognized (3, 5, 20, 27, 28).

When we compared the HCV NS3<sub>987-1133</sub> sequence of the infectious source with sequences that we isolated from the sera of persistently infected, HLA-A2-positive patients 18 years after the single-source outbreak, we found the highest ratio of nonsynonymous to synonymous HCV mutations at amino acid position NS3<sub>1082</sub> (Figure 1). Position NS3<sub>1082</sub> was located directly adjacent to the carboxyterminus of an HLA-A2-restricted CD8<sup>+</sup> T cell epitope recognized by recovered patients of this (20) and other cohorts (4, 20–25). Whereas 54 molecular clones from 3 independent PCRs of the infectious source encoded a tyrosine (Y) in position NS3<sub>1082</sub>, serum isolates from 15 of 21 (71%) HLA-A2-positive, persistently infected patients encoded a phenylalanine (F) at position NS3<sub>1082</sub> (Table 1). Because the Y/F mutant was also observed in a substantial number of HLA-A2-negative, persistently infected patients (11 of 24 [46%];  $P = 0.07$ ; data not shown), the epidemiological data alone did not indicate whether HLA-A2-restricted T cell selection pressure could have contributed to the viral sequence evolution in this patient cohort. We therefore decided to analyze the molecular and immunological effects of the Y/F mutation *in vitro* by studying proteasomal processing of antigen and *in vivo* by studying its effect on T cell priming in an HLA-transgenic mouse model.

*The Y/F mutation at residue NS3<sub>1082</sub> impairs carboxyterminal processing of the NS3<sub>1073-1081</sub> epitope.* Most MHC class I-restricted peptides are liberated from antigenic precursor sequences by the 20S core particle of the proteasome. To analyze whether the NS3<sub>1082</sub> Y/F substitution affected proteasomal processing of the NS3<sub>1073-1081</sub> epitope, we built on our previous demonstration that proteasome-dependent *in vitro*

processing of epitope-harboring polypeptides reflects the *in vivo* situation with high fidelity (10, 12, 29, 30) and compared proteasome-dependent *in vitro* processing of the NS3 wild-type polypeptide, designated NS3(Wt)<sub>1062-1095</sub>, with processing of the NS3 mutant polypeptide, designated NS3(Mut)<sub>1062-1095</sub>. For this purpose, immunoproteasomes were purified from IFN- $\gamma$ -stimulated HepG2 human hepatoma cells and from murine mouse embryonal cells-217 (MEC-217) transfected with the immunoproteasome subunits LMP2, LMP7 and MECL-1 (10). Constitutive proteasomes were purified from unstimulated HepG2 cells and from murine MEC-18 cells.

Synthetic NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptides (Figure 2a) were then incubated with 20S immunoproteasomes. The digestion products were separated and analyzed by reverse phase-HPLC and mass spectrometry. NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptide substrates were digested with the same kinetics with nearly 40–50% turn-over of each substrate within 4–8 hours (Figure 2B). Because longer digestion times resulted in secondary cleavage of processing intermediates, 4-hour and 8-hour digestion times were considered optimal for further biochemical analyses. Processing with constitutive proteasomes yielded the same qualitative results but lower amounts of digest product than processing with immunoproteasomes (not shown).

In contrast to the substrates, the relative abundance of the NS3<sub>1073-1081</sub> epitope could not be precisely determined because the epitope's 2 cysteine residues formed aggregates via disulfide bonds. These aggregates interfered with reliable identification of the NS3<sub>1073-1081</sub> epitope and its immediate precursors by mass spectrometry. As an indirect measure of the generation of the NS3<sub>1073-1081</sub> epitope, we therefore determined the relative abundance of those cleavage products that flanked its amino- and carboxyterminus. Cleavage product NS3<sub>1062-1070</sub>, for example, flanked the amino-terminus of the NS3<sub>1073-1081</sub> epitope. Cleavage product



**Table 1**

HCV NS3<sub>1062-1095</sub> sequence of the infectious source and HLA-A2<sup>+</sup> HCV-infected patients

Experiment/patient	HCV <sub>NS3 1062-1094</sub> amino acid sequence	Analyzed molecular clones/ PCR product
<b>Infectious source</b>		
PCR 1	VSTATQSFLATCVNGVCWTVYHGAGSKTLGPKG	16 clones
	-----E-----	1 clone
	-----R-----	1 clone
	-----L-----	1 clone
	-----A-----	1 clone
	-----A-----	1 clone
PCR 2	-----S-----	14 clones
	-----L-----	1 clone
PCR 3	-----A-----	12 clones
	-----R-----	2 clones
	-----T-----	1 clone
	-----R-----	1 clone
<b>HLA-A2<sup>+</sup>, persistently infected HCV patients</b>		
Patient 1	-----A-----	17 clones
	-----A-----	1 clone
Patient 2	-----A-----	12 clones
	-----E-----	1 clone
	-----D-----	1 clone
Patient 3	-----S-----	PCR product
Patient 4	-----F-----	PCR product
Patient 5	-----L-----	PCR product
Patient 6	-----F-----	15 clones
Patient 7	-----F-----	1 clone
Patient 8	-----F-----	19 clones
	-----F-S-----	1 clone
Patient 9	-----F-----	12 clones
	-----FQ-----	1 clone
Patient 10	-----F-----	10 clones
Patient 11	-----F-----	14 clones
	-----S-----	1 clone
	-----A-----	1 clone
	-----V-----	1 clone
Patient 12	-----F-----	PCR product
Patient 13	-----F-----	PCR product
Patient 14	-----F-----	PCR product
Patient 15	-----F-----	PCR product
Patient 16	-----F-----	PCR product
Patient 17	-----F-----	PCR product
Patient 18	-----F-----	PCR product
Patient 19	-----S-----	PCR product
Patient 20	-----I-----	PCR product
Patient 21	-----F-----	PCR product

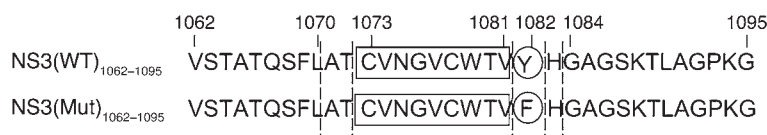
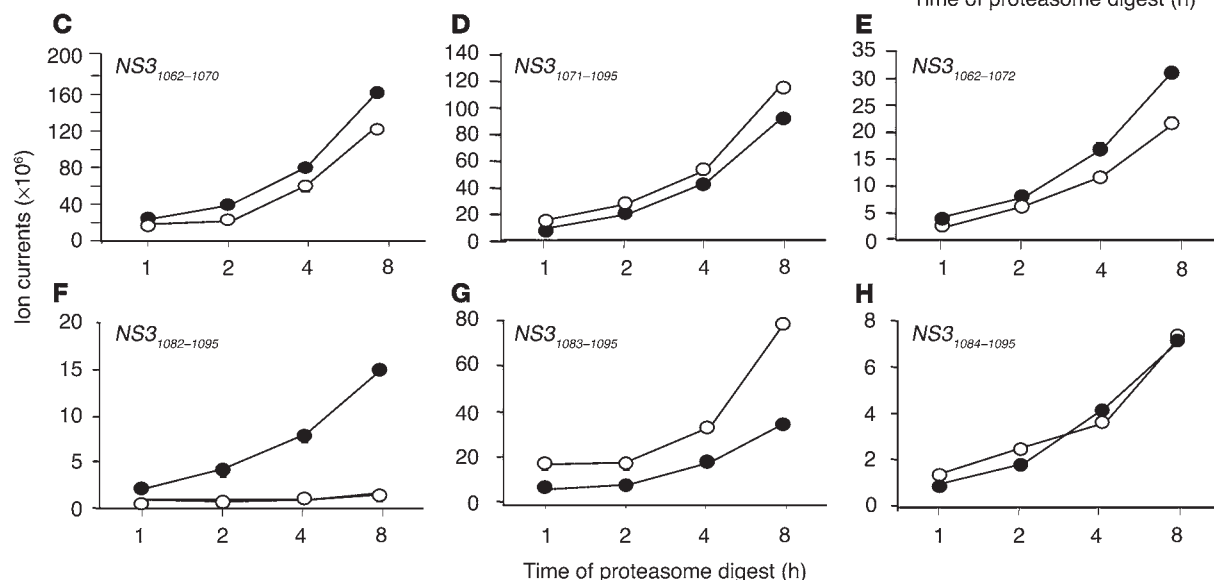
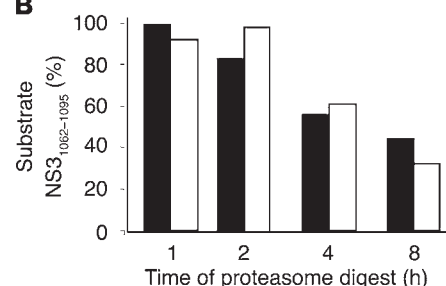
an amino-terminal elongation of the NS3<sub>1073-1081</sub> epitope by 2 amino acids.

A second cleavage product that flanked the amino-terminus of the NS3<sub>1073-1081</sub> epitope was peptide NS3<sub>1062-1072</sub>. As indicated in Figure 2E, cleavage product NS3<sub>1062-1072</sub> was also liberated from both the NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptide substrates in comparable amounts (Figure 2E), indicating that the proteasome cut both the NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptide between amino acid positions 1072 and 1073 as indicated by the dotted line in Figure 2A. This proteasomal cut resulted in the generation of the correct amino-terminus of the NS3<sub>1073-1081</sub> epitope, irrespective of the presence or absence of the NS3<sub>1082</sub> mutation. The demonstration that the NS3<sub>1073-1081</sub> epitope and its amino-terminally elongated forms were generated at the same time is consistent with previous findings for other epitopes (31, 32). In cases of amino-terminally elongated peptides, the precise amino-terminus has been shown to be further defined by postproteasomal trimming by aminoexopeptidases (31, 32).

In contrast to the amino-terminus, the correct carboxyterminus of the NS3<sub>1073-1081</sub> epitope was generated only from the wild-type polypeptide. Figure 2 (F, G, and H) shows the relative abundance of those cleavage products whose generation defined the epitope's carboxyterminus at amino acid position NS3<sub>1082</sub>. The generation of cleavage product NS3<sub>1082-1095</sub> indicated the correct processing of the epitope's carboxyterminus with a proteasomal cut between amino acid positions 1081 and 1082 (Figure 2F). Importantly, cleavage product NS3<sub>1082-1095</sub> was only liberated from the NS3(Wt)<sub>1062-1095</sub> and not from the NS3(Mut)<sub>1062-1095</sub> polypeptide substrate (Figure 2F). In contrast, cleavage product NS3<sub>1083-1095</sub> was liberated from the NS3(Mut)<sub>1062-1095</sub> more efficiently than from the NS3(Wt)<sub>1062-1095</sub> polypeptide substrate (Figure 2G). This result indicated that, in the presence of the NS3<sub>1082</sub> mutation, the proteasome cut between amino acid positions 1082 and 1083 rather than between amino acid positions 1081 and 1082 and thus generated

NS3<sub>1062-1070</sub> was generated from both the NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptide substrates with comparable efficiency (Figure 2C). Generation of cleavage product NS3<sub>1062-1070</sub> was associated with the generation of the complementary cleavage product NS3<sub>1071-1095</sub> (Figure 2D), consistent with a proteasomal cut between amino acid positions 1070 and 1071 in both the NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptide substrates (see dotted line in Figure 2A). This proteasomal cut resulted in

NS3<sub>1062-1070</sub> was generated from both the NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptide substrates with comparable efficiency (Figure 2C). Generation of cleavage product NS3<sub>1062-1070</sub> was associated with the generation of the complementary cleavage product NS3<sub>1071-1095</sub> (Figure 2D), consistent with a proteasomal cut between amino acid positions 1070 and 1071 in both the NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptide substrates (see dotted line in Figure 2A). This proteasomal cut resulted in

**A****B****Figure 2**

Digestion of NS3(Wt)<sub>1062–1095</sub> polypeptide and NS3(Mut)<sub>1062–1095</sub> polypeptide with purified 20S immunoproteasomes. **(A)** The amino acid sequences of the 34-mer NS3(Wt)<sub>1062–1095</sub> and NS3(Mut)<sub>1062–1095</sub> polypeptides are shown in single-letter code. The HLA-A2 restricted CD8<sup>+</sup> T cell epitope NS3<sub>1073–1081</sub> is framed, and amino acid position NS3<sub>1082</sub> containing the wild-type tyrosine or the mutant phenylalanine is circled. The dashed lines indicate the location of proteasomal cuts as deduced from the data shown in panels C–H. **(B)** NS3(Wt)<sub>1062–1095</sub> polypeptide (black bars) and NS3(Mut)<sub>1062–1095</sub> polypeptide (white bars) were digested for 1, 2, 4, and 8 hours with 20S immunoproteasomes. Kinetic analysis of polypeptide substrate turnover is indicated. **(C–H)** Kinetic analysis of cleavage product generation. Relative abundances of cleavage products derived from immunoproteasomal digestion of NS3(Wt)<sub>1062–1095</sub> polypeptide (filled circles) and NS3(Mut)<sub>1062–1095</sub> polypeptide (open circles) are plotted. The relative abundance of the NS3<sub>1073–1081</sub> epitope could not be assessed due to formation of disulfide bonds that interfered with mass spectrometry analysis.

a carboxyterminally elongated NS3 epitope. Finally, cleavage product NS3<sub>1084–1095</sub> was liberated from both the NS3(Wt)<sub>1062–1095</sub> and NS3(Mut)<sub>1062–1095</sub> polypeptide substrates in comparable amounts (Figure 2H), indicating that the proteasomal cut between amino acid 1083 and 1084 was not affected by the NS3<sub>1082</sub> mutation.

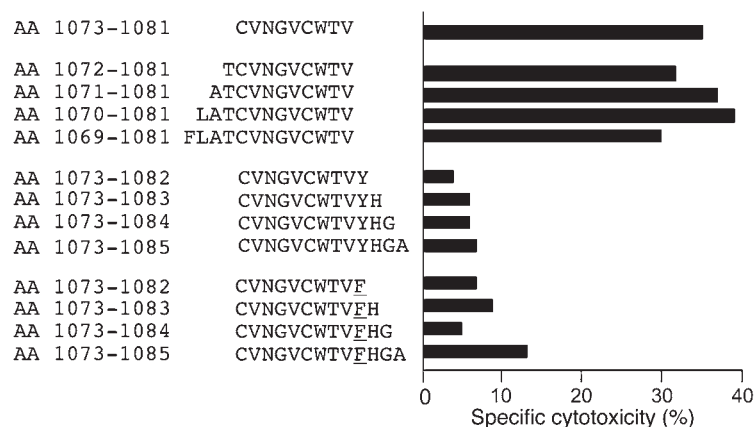
In summary, an incorrect carboxyterminus of the NS3<sub>1073–1081</sub> epitope appeared to be generated from the NS3(Mut)<sub>1062–1095</sub> but not from the NS3(Wt)<sub>1062–1095</sub> polypeptide substrate.

NS3<sub>1073–1081</sub>-specific CD8<sup>+</sup> T cells recognize aminoterminally, but not carboxyterminally elongated forms of the minimal optimal epitope. To study recognition of the processing products by CD8<sup>+</sup> T cells, we generated NS3<sub>1073–1081</sub>-specific CD8<sup>+</sup> T cell lines from the blood of HCV-recovered patients. These T cell lines recognized target cells pulsed with the minimal optimal NS3<sub>1073–1081</sub>-epitope in a standard cytotoxicity assay (Figure 3). Epitope-specific T cell lines also recognized the corresponding aminoterminally elongated peptides, but not the carboxyterminally elongated mutant and wild-type peptides (Figure 3), thereby confirming that correct carboxyterminal cleavage by the proteasome was indispensable (33, 34).

*Direct biochemical and immunological detection of the NS3<sub>1073–1081</sub> epitope in proteasomal digests.* To directly assess and quantitate the in vitro generation of the NS3<sub>1073–1081</sub> epitope, it was necessary to prevent the formation of disulfide bonds between the cysteine residues. We therefore synthesized a serine variant of the epitope with a cysteine to serine exchange at position NS3<sub>1073</sub>. This NS3<sub>1073–1081</sub> serine variant was equally well recognized by cytotoxic T cells as the original NS3<sub>1073–1081</sub> epitope (Figure 4A).

When the corresponding NS3(Wt)<sub>1062–1095</sub> and NS3(Mut)<sub>1062–1095</sub> polypeptides with a serine in position NS3<sub>1073</sub> were subjected to digestion by constitutive proteasomes, the production of the NS3<sub>1073–1081</sub> serine variant could be directly assessed by mass spectrometry. As demonstrated in Figure 4B, a significantly larger amount of the NS3<sub>1073–1081</sub> SVNGVCWTV epitope was generated from the NS3(Wt)<sub>1062–1095</sub> polypeptide than from the NS3(Mut)<sub>1062–1095</sub> polypeptide. For immunological analysis, the complete 8-hour proteasomal digests were then loaded onto transporter associated with antigen processing-deficient (TAP-deficient), T2-target cells and tested for recognition by NS3<sub>1073–1081</sub>-specific, cytotoxic T cell



**Figure 3**

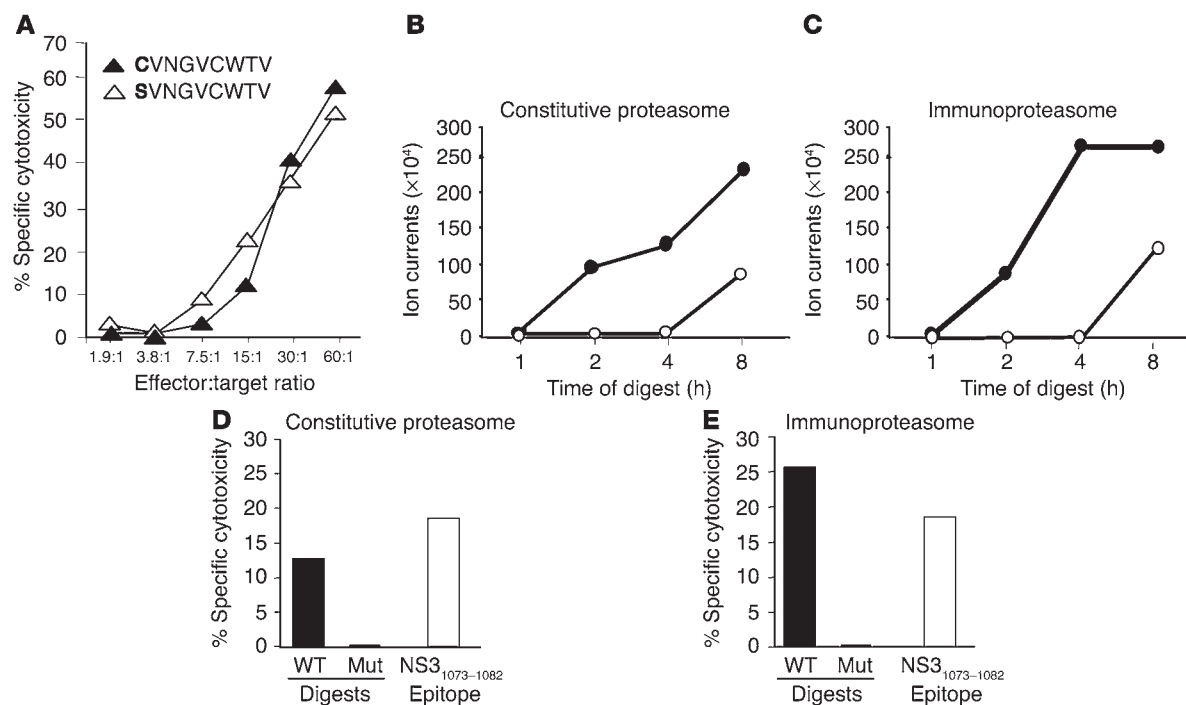
NS3<sub>1073-1081</sub> epitope-specific CD8<sup>+</sup> T cells recognize aminoterminally extended, but not carboxyterminally extended, peptides of the minimal optimal NS3<sub>1073-1081</sub> epitope. NS3<sub>1073-1081</sub>-specific CD8<sup>+</sup> T cells were expanded from PBMCs of an HCV-recovered patient by several weeks of peptide stimulation and tested against amino- and carboxyterminally extended peptides in a standard <sup>51</sup>Cr-release assay. The mutant sequence at AA position 1082 is underlined.

lines. Only the wild-type and not the mutant polypeptide digests were recognized by NS3<sub>1073-1081</sub>-specific cytotoxic T cells (Figure 4D), thus confirming the biochemical data in Figure 2.

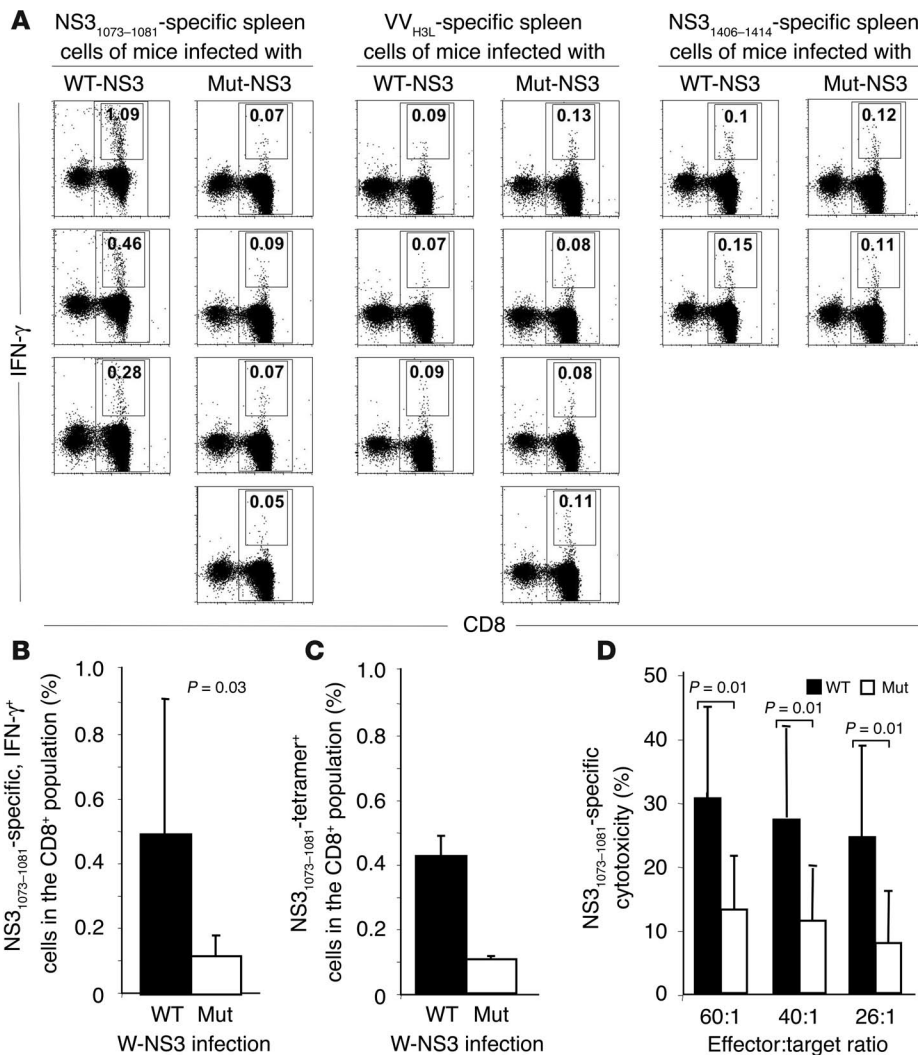
The same qualitative results were obtained when NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> polypeptides were digested with immunoproteasome instead of constitutive proteasome. Again, a significantly larger amount of the NS3<sub>1073-1081</sub> SVNGVCWTV epitope was generated from NS3(Wt)<sub>1062-1095</sub> than from NS3(Mut)<sub>1062-1095</sub> polypeptide (Figure 4, C and E). Overall, the immunoproteasome appeared to digest the wild-type polypeptide more rapidly than the constitutive proteasome did, as indicated by a plateau-phase

of epitope liberation from the wild-type polypeptide in the biochemical analysis (Figure 4C) and by the higher cytotoxicity in the immunological analysis (Figure 4E).

*The HCV NS3<sub>1082</sub> Y/F mutant reduces the induction of HCV NS3<sub>1073-1081</sub>-specific CD8<sup>+</sup> T cells in HLA-A2 transgenic mice.* To analyze whether the altered proteasomal cleavage of the NS3<sub>1082</sub> Y/F mutant affected the generation of the NS3<sub>1073-1081</sub> epitope in vivo, we employed a humanized mouse model. Specifically, we used transgenic mice that expressed the  $\alpha$ 1 and  $\alpha$ 2 chains of the human HLA-A2 molecule and the  $\alpha$ 3 chain of the murine K<sup>d</sup> molecule (35). Upon immunization, these mice generate T cells against the same HLA-

**Figure 4**

Biochemical and immunological analysis of proteasomal digests. (A) NS3<sub>1073-1081</sub>-specific CD8<sup>+</sup> T cells lyse T2 cells loaded with either the NS3<sub>1073-1081</sub> serine variant SVNGVCWTV or the NS3<sub>1073-1081</sub> epitope CVNGVCWTV. (B–C) Generation of NS3<sub>1073-1081</sub> serine variant SVNGVCWTV from NS3(Wt)<sub>1062-1095</sub> (filled circles) and NS3(Mut)<sub>1062-1095</sub> (open circles) serine variant polypeptides by digestion with constitutive 20S proteasome (B) and by digestion with 20S immunoproteasome (C). (D–E) 8-hour-proteasome (D) and immunoproteasome (E) digests of NS3(Wt)<sub>1062-1095</sub> and NS3(Mut)<sub>1062-1095</sub> serine variant polypeptides were loaded onto TAP-deficient T2-target cells and incubated with NS3<sub>1073-1081</sub> epitope-specific, cytotoxic T cells at an effector to target ratio of 13 to 1. For comparison,  $10^{-5}$  M NS3<sub>1073-1081</sub> serine variant was loaded onto T2 target cells. Only wild-type and not the mutant polypeptide digests were recognized by NS3<sub>1073-1081</sub> epitope-specific cytotoxic T cells.



**Figure 5** Immunization of HLA-A2 transgenic mice with the wild-type HCV NS3 sequence induces more IFN- $\gamma$ -secreting and cytotoxic HCV NS3<sub>1073-1081</sub>-specific CD8 T cells than immunization with the mutant HCV NS3 sequence. (A and B) Ex vivo analysis of NS3<sub>1073-1081</sub>-specific, IFN- $\gamma$ -producing CD8 T cells demonstrated a significantly greater response in mice immunized with wild-type than of mice immunized with mutant NS3 encoding vaccinia virus. In contrast, the response against the vaccinia virus<sub>H3L</sub> (VV<sub>H3L</sub>) epitope and the HCV NS3<sub>1406-1414</sub> epitope did not differ between both groups of mice. (A) Dot plots from individual mice tested in the same experiment. (B) Mean and standard deviation of the results of all 8 mice per group. (C) Frequency of NS3<sub>1073-1081</sub>-tetramer-specific T cells is higher in mice immunized with wild-type than in mice immunized with mutant HCV NS3 sequences. Mean and standard deviation of the results of 8 mice per group are shown. (D) NS3<sub>1073-1081</sub>-specific T cell lines from mice immunized with wild-type HCV NS3 sequences (WT) display greater cytotoxicity than those derived from mice immunized with mutant HCV NS3 (Mut). Mean and standard deviation of the results of 11 mice per group are shown.

A2-restricted epitopes as HLA-A2-positive humans (36). In contrast to what occurs in human studies, however, this mouse model allows the induction of T cells in the context of a single, defined HLA molecule and a single, defined viral sequence. Therefore, the HLA-A2-transgenic mouse model is not subject to the influences of additional factors such as selection pressure in the context of other HLA molecules and epitopes and/or preservation of viral replication fitness as may be operating in vivo in infected humans.

To take advantage of this model, we immunized HLA-A2-transgenic mice with vaccinia viruses that encoded full-length HCV NS3 sequences with either the Y wild type or the F mutant at amino acid position NS3<sub>1082</sub>. Two weeks after immunization, spleen cells were isolated, and the frequency of NS3<sub>1073-1081</sub>-specific T cells was assessed by ex vivo intracellular IFN- $\gamma$ -staining and by tetramer analysis. As shown in Figure 5A for individual mice and in Figure 5, B and C, for all mice, the frequency of NS3<sub>1073-1081</sub>-specific, IFN- $\gamma$ -producing and tetramer<sup>+</sup> cells was significantly higher in mice infected with wild-type than in those infected with mutant NS3 encoding virus. In contrast, the frequency of CD8<sup>+</sup> cells that recognized either a vaccinia virus epitope or an unrelated HCV NS3 epitope did not differ between both groups of mice, thus confirming the specificity of the observation.

In separate experiments, additional immunizations with recombinant DNA-expression vectors were performed to increase the number of NS3<sub>1073-1081</sub>-specific T cells and to establish T cell lines suitable for cytotoxicity analyses. At all effector/target ratios, cytotoxic T cell responses of mice that had been immunized with wild-type NS3 sequences were significantly stronger than cytotoxic T cell responses of mice immunized with mutant NS3 sequences (Figure 5D). Collectively, these results demonstrate that the NS3<sub>1082</sub> Y/F substitution reduced the generation of the NS3<sub>1073-1081</sub> epitope in vitro when polypeptides were digested with purified 20S proteasomes. In addition, the NS3<sub>1082</sub> substitution impaired the induction of epitope-specific T cells in vivo when full-length NS3 protein was endogenously expressed, ubiquitinated, and processed by the 26S proteasome in the presence of additional cytosolic proteases.

## Discussion

Most MHC class I ligands are liberated from strings of polypeptides and ubiquitinated proteins by the proteasome, the main cytosolic protease (13). Whereas the amino-terminus of each epitope can be further defined by postproteasomal aminopeptidases (31, 32, 37), the carboxyterminus needs to be defined precisely with the first



cut (33, 34). Studying a single source outbreak of HCV, we identified an HCV mutation that interfered with the correct carboxyterminal cleavage of an immunodominant, HLA-A2 restricted HCV epitope from its mutated polypeptide precursor.

The emergence of viral mutations in immunogenic sequences has long been discussed as a potential immune escape mechanism. As regards HCV, sequences that do not bind to the MHC and/or the T cell receptor and thus are not recognized by HCV-specific CD8<sup>+</sup> T cells have been observed in chimpanzees and in humans (15, 16, 18). In addition, mutations that generate partial agonists or antagonists to the T cell receptor and downregulate wild-type-specific T cell responses have been described in HCV (18) as well as in hepatitis B virus (HBV) (38) and HIV infections (39). Perhaps even more efficient mechanisms of viral escape are mutations in epitope-flanking residues that interfere with antigen processing and presentation of MHC class I-restricted epitopes because, in these cases, the induction phase rather than the effector phase of HCV-specific T cell responses can be impaired. Indeed, the literature provides several examples showing that amino acid residues in the flanking regions of T cell epitopes impair proteasomal processing of those epitopes (40–43). On the other hand, there are also examples showing that extensive sequence changes in the flanking regions of other immunodominant CD8 T cell epitopes do not influence antigen processing (44). In our study, we observed impairment of antigen processing by an exchange of two very similar amino acids. Although the Y/F substitution is a conservative one, it impaired correct carboxyterminal cleavage of the NS3<sub>1073–1081</sub> epitope not only by constitutive proteasomes, but also by immunoproteasomes. Importantly, the same effects were observed in vitro when polypeptides were digested with purified 20S proteasomes and in vivo when full length HCV NS3 protein was endogenously expressed in an animal model, ubiquitinated, and processed by the 26S proteasome in the presence of additional cytosolic proteases. Thus, the data demonstrate a mechanism by which a conservative HCV mutation can interfere with the induction of epitope-specific CD8<sup>+</sup> T cells.

The observation that this specific HCV mutation was also found in a substantial number of HLA-A2-negative patients is consistent with a recent report on an HIV mutation in an HLA-B51-restricted epitope, which was also less frequent in HLA-B\*51-negative than in HLA-B\*51-positive persons, but not completely absent in HLA-B\*51-negative persons. Overall, 29% of HLA-B\*51-negative persons carried the HIV mutation as compared to 98% of HLA-B\*51-positive persons (43). Notably, with more than 400 patients enrolled, the HIV study was much larger than our study, and differences in the prevalence of the HIV mutation between patient subgroups were remarkable and statistically significant (43). In the HIV study as well as in our study, however, the presence of the mutation in a subpopulation of patients without the relevant HLA haplotype is consistent with the influence of multiple selection forces that drive the evolution of viral sequences in humans. These selection forces include pressure on additional, overlapping, or adjacent T cell epitopes that are presented in the context of other MHC class I and II alleles as well as selection pressure to preserve viral replication fitness (45). Because the HCV NS3<sub>1073–1082</sub> sequence is located directly downstream of the HCV protease domain, it is, for example, possible that the Y/F mutation affects HCV replication and was therefore also found in HLA-A2-negative patients. For these reasons, the HLA-A2-transgenic mouse model provides a valuable tool for analyzing the effect of

a single mutation on the induction of epitope-specific CD8<sup>+</sup> cells in the context of a single, defined HLA molecule.

Although this study demonstrated a mechanism of altered antigen processing and impaired induction of HCV-specific T cells, significantly larger, population-based studies will be required to analyze the contribution of this mechanism to the overall selection pressure that drives HCV sequence evolution in infected patients. As a starting point for these studies, mutations in the flanking region of this particular HCV NS3<sub>1073–1081</sub> epitope are intriguing for several reasons. In addition to being the single most vigorously recognized CD8<sup>+</sup> T cell epitope in all published studies (4, 20–25), the NS3<sub>1073–1081</sub> epitope is one of only a few described epitopes that are recognized by circulating T cells as well as by nonspecifically expanded intrahepatic T cells (46). Moreover, it is also one of only two epitopes for which a TCR antagonist based on an intraepitope mutation has been described in persistently infected patients (18). The Y/F mutation in the flanking region of the epitope described here should, however, not be regarded as a main cause of HCV persistence. Rather, it should be regarded as an example of a viral escape mechanism that might also occur in the flanking regions of other CD4<sup>+</sup> and CD8<sup>+</sup> T cell epitopes. If many of these mutations occur throughout the HCV polyprotein, they may collectively contribute to the evolution of HCV quasi-species in persistently infected patients and to the characteristic weakness of the HCV-specific immune response.

## Methods

**Clinical samples.** Sera of patients with persistent HCV infection were analyzed 18 years after an accidental single-source outbreak (genotype 1b, AJ32996) due to a contaminated anti-D immunoglobulin (20, 26). The patients gave informed consent to this analysis. Patient samples were analyzed at Medizinische Hochschule Hannover (MHH) under a protocol approved by the MHH Ethics Committee. At the time of analysis, none of the patients had developed liver cirrhosis. HCV persistence was defined by detection of serum HCV RNA by RT-PCR, and by detection of HCV antibodies by enzyme immunoassay (HCV Version 3.0; Abbott Diagnostika GmbH, Wiesbaden, Germany). HLA typing was performed with Terasaki HLA-typing trays (One Lambda Inc., Canoga Park, California, USA).

**Peptides.** The wild-type NS3(Wt)<sub>1062–1095</sub> VSTATQS-FLATCVNGVCWTVYHGAGSKTLGAPKG polypeptide, the mutant NS3(Mut)<sub>1062–1095</sub> VSTATQSFLATCVNGVCWTVFHGAGSKTLGAPKG polypeptide, the NS3<sub>1062–1095</sub> serine variants VSTATQSFLATSVNGVCWTVYHGAGSKTLGAPKG and VSTATQSFLATSVNGVCWTVFHGAGSKTLGAPKG, the NS3<sub>1073–1081</sub> epitope CVNGVCWTV and the corresponding carboxyterminally and/or amino-terminally elongated peptides, the NS3<sub>1073–1081</sub> serine variant SVNGVCWTV, and the control peptides vaccinia virus<sub>H3L</sub> SLSAYIIRV (47) and HCV NS3<sub>1406–1415</sub> KLVALGINAV were synthesized using standard Fmoc methodology on an Applied Biosystems 433A automated synthesizer at >90% purity (Applied Biosystems, Darmstadt, Germany).

**Detection and sequencing of HCV.** Total RNA was extracted from 140 µl sera using QIAamp Viral RNA Kit (QIAGEN GmbH, Hilden, Germany), and reverse transcription was performed with Superscript II Reverse Transcriptase (Gibco BRL, Grand Island, New York, USA) and random hexamers (48, 49). HCV sequences from the highly conserved 5' untranslated region were amplified with Taq Polymerase (Gibco BRL) in a nested PCR with prim-





ers HCV43S (CCCTGTGAGGAACT[AT]CTGTCTTCACGC) and HCV318AS (GGTGCACGGTCTACGAGACCT) and nested primers HCV78S (TCTAGCCATGGCGTTAGTCAG[CT]GA) and HCV288AS (CACTCGCAAGCACCTATCAGGCAGT) (BioteZ Berlin-Buch GmbH, Berlin, Germany) in 1 cycle with 5 minutes at 93°C, 2 minutes at 52°C, 3 minutes at 72°C; in 35 cycles with 1 minute at 93°C, 1 minute at 52°C, 2 minutes at 72°C; and in 1 cycle with 4 minutes at 72°C. PCR products were visualized on an ethidium bromide-stained 1.5% agarose gel.

For sequencing analysis, reverse transcription of RNA isolated from the inoculum was performed with the HCV-specific primer NS3as (CAGCATGCCTCGTGACCA); reverse transcription of RNA isolated from the serum of persistently infected patients was performed with Superscript II Reverse Transcriptase (Gibco BRL) and random hexamers as previously described (48, 49). The NS3<sub>987-1133</sub> region containing the NS3<sub>1073-1081</sub> epitope was amplified with primers NS3s (GAGGCCACTATGTCCAAATG) and NS3as (CAGCATGCCTCGTGACCA) and nested primers NS3s-nested (GTAGAGCCCGTCGTCTTCTC) and NS3as-nested (GCCTCGTGACCAAGTAAA-GG). PCR conditions for the outer primer pair were 1 cycle with 2 minutes at 94°C; 30 cycles with 30 seconds at 94°C, 90 seconds at 50°C, and 2 minutes at 72°C; and 1 cycle with 4 minutes at 72°C. PCR conditions for the inner primer pair were 1 cycle with 2 minutes at 94°C; 30 cycles with 30 seconds at 94°C, 90 seconds at 59°C, and 2 minutes at 72°C; and 1 cycle with 4 minutes at 72°C. PCR products were either sequenced directly using the ABI PRISM BigDye Terminator Cycle Ready Reaction Kit and the ABI PRISM 310 sequencer (PerkinElmer, Rodgau-Jügesheim, Germany) or cloned into pCRII Vector (Topo TA Cloning Kit; Invitrogen, Carlsbad, California, USA) for sequencing of molecular clones. Sequence analysis was performed with software Factura and Sequence Navigator (PerkinElmer).

*Generation of recombinant vaccinia viruses encoding wild-type and mutant HCV NS3.* The HCV clone HCV-AD78P1 (Genbank number AJ132997) (50) was used as a template to amplify the full-length NS3 sequence by PCR using primers NS3FL-s (CCGCTAGC-CACCATGGCGCCCATCAGGCCTATTCC; nt 3081-3101) and NS3FL-as (CCGCGGCCGCTTAGGTGACGACCTCCAGGTCAGC; nt 4974-4954) under the following PCR conditions: 1 cycle with 2 minutes at 95°C; 5 cycles with 30 seconds at 94°C, 1 minute at 52°C, and 4 minutes at 72°C; followed by 25 cycles with 30 seconds at 94°C, 1 minute at 60°C, and 4 minutes at 72°C; and 1 cycle with 4 minutes at 72°C. The primer pair included a KpnI (5') and NotI (3') restriction site, respectively, for cloning into the pEF1/myc-His vector (Invitrogen). Site-directed mutagenesis was performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA) and primers GCTGGAGTGTCTACCATG-GCGCTGGC and GCCAGCGCCATGGTAGACAGTCCAGC to substitute the tyrosine for phenylalanine at position NS3<sub>1082</sub>. NS3-sequences were then subcloned into plasmid p7.5K131 (51) using EcoRI restriction sites to generate the recombinant vaccinia viruses VV<sub>NS3-Wt</sub> and VV<sub>NS3-Mut</sub> by homologous recombination with the Copenhagen strain and selection on 143TK<sup>-</sup> cells. Synthesis of HCV-NS3-specific mRNA was confirmed by Northern blot analysis of CV-1 cells infected with vaccinia viruses (MOI 10) using radioactively labeled HCV NS3-cDNA and standard techniques (52). HCV-NS3 expression of wild-type and mutant NS3-encoding vaccinia virus was comparable as determined by flow cytometry (not shown).

*Proteasome purification and peptide digestion.* Constitutive 20S proteasomes were isolated from HepG2 human hepatoma cells

and murine MEC-18 cells. Immunoproteasomes were isolated from HepG2 that had been cultured with 200 U/l human IFN- $\gamma$  (Roche Diagnostics GmbH, Mannheim, Germany) for 72 hours and from MEC-18 cells that had been transfected with LMP2, LMP7, and MECL-1 under a tetracycline-regulated promoter (10). The purity of isolated proteasomes was greater than 90% (11). Twenty micrograms of wild-type and mutant NS3<sub>1062-1095</sub> peptides were incubated with 2  $\mu$ g purified proteasomes in 150  $\mu$ l assay buffer (20 mM Hepes/KOH, pH 7.8, 2 mM MgAc<sub>2</sub>, 1 mM dithiothreitol) at 37°C. The reaction was terminated by the addition of 0.1% trifluoroacetic acid (TFA). Forty microliters of the digests were separated by reversed-phase chromatography on a  $\mu$ RPC C2/C18 2.1/10 column (Pharmacia Biotech, Freiburg, Germany) and analyzed online with an ion trap mass spectrometer (LCQ; Electron Corp., Dreieich, Germany) with an electrospray ion source. Peptides were identified by tandem mass spectrometry (MS/MS) experiments and the amount of generated NS3<sub>1073-1081</sub> epitope was calculated by comparison to a defined amount of synthetic NS3<sub>1073-1081</sub> peptide.

*In vivo induction of HCV-specific CD8<sup>+</sup> T cells in HLA-A2-transgenic mice.* AAD mice, which express the  $\alpha$ 1 and  $\alpha$ 2 domains of the HLA-A2.1 molecule and the  $\alpha$ 3 domain of the murine H-2D<sup>d</sup> molecule (35), were used to test the in vivo effect of the HCV mutation on the induction of NS3<sub>1073-1081</sub>-specific T cells. The animal protocol was approved by the National Institute for Diabetes, Digestive and Kidney Diseases (NIDDK) Animal Care and Use Committee.

For ex vivo analysis of NS3<sub>1073-1081</sub>-specific T cells, 6- to 8-week-old AAD mice were intraperitoneally injected with 10<sup>7</sup> PFU recombinant vaccinia virus (VV<sub>NS3-Wt</sub> and VV<sub>NS3-Mut</sub> respectively) in 200  $\mu$ l PBS. Spleens were isolated two weeks after immunization, injected with 400  $\mu$ g/ml of Liberase CI (Roche Diagnostics, Indianapolis, Indiana, USA), incubated at 37°C for 30 min and forced through a cell strainer (Falcon; BD Biosciences, Franklin Lakes, New Jersey). Single-cell suspensions were subsequently incubated with purified anti-mouse CD16/CD32 (Fc $\gamma$ III/IIR; BD Pharmingen, San Diego, California, USA) for 15 minutes at 4°C, then washed with PBS/2% FBS and stained with the HLA-A2/HCV NS3<sub>1073</sub> tetramer (NIAID Tetramer Facility, Atlanta, Georgia, USA) for 30 minutes at room temperature. After two washes, cells were stained with FITC-conjugated anti-CD8 for an additional 30 minutes at 4°C, washed again, and resuspended in 500 l PBS/0.5% paraformaldehyde (PFA) for flow cytometry.

For analysis of cytokine production, CD8<sup>+</sup> T cells were isolated from spleen cells using MACS CD8<sup>+</sup> T Cell Isolation Kit and Columns (Miltenyi Biotec, Auburn, California, USA), according to the manufacturer's instructions. C1R-AAD cells (10<sup>6</sup>) (35) that had been pulsed overnight with 10 g/ml of either HCV NS3<sub>1073-1081</sub>, VV H3L<sub>35-43</sub>, or HCV NS3<sub>1406-1415</sub> peptide were used to stimulate 2  $\times$  10<sup>6</sup> purified CD8<sup>+</sup> T cells for 12 hours in 1 ml RPMI1640 containing 10% fetal bovine serum (BioWhittaker, Walkersville, Maryland, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ M 2-mercaptoethanol (standard medium), additionally supplemented with 50 U/ml IL-2, 10 g/ml brefeldin A (BD Pharmingen), 2 l anti-CD28, and 2 l anti-CD49d. Cells were then pelleted, washed with PBS/2% FBS, and stained at 4°C for 30 minutes with FITC-conjugated anti-CD8 (BD Pharmingen). After two washes in PBS, cells were fixed with Cytofix/Cytoperm (BD Pharmingen) at 4°C for 30 minutes. Thereafter, cells were washed twice with 1 $\times$  Perm/Wash buffer (BD Pharmingen) and stained at 4°C for 30 minutes with PE-conjugated anti-IFN- $\gamma$  or



PE-conjugated IgG1 isotype control. After one additional wash in 1× Perm/Wash buffer, cells were resuspended in PBS and analyzed using a Becton-Dickinson FACSCalibur with CellQuest (BD, San Jose, California, USA) and FlowJo software (Tree Star, San Carlos, California, USA). At least 10,000 events were acquired in a forward and side-scatter gate set to exclude cell debris.

For the generation of T cell lines and for in vitro analysis of cytotoxicity, mice were immunized twice with  $10^7$  PFU recombinant vaccinia virus (VV<sub>NS3-Wt</sub> or VV<sub>NS3-Mut</sub>) in 200 µl PBS at 4-week intervals. An additional intramuscular immunization with NS3-Wt and NS3-Mut encoding pEF1/myc-His plasmids (Invitrogen) was performed prior to the vaccinia virus immunization in some experiments, but did not further enhance the NS3<sub>1073-1081</sub>-specific T cell response. Spleen cells were harvested 7 days after the last immunization and stimulated in T-25 flasks ( $3 \times 10^7$  cells/flask) in standard medium containing 50 µg/ml synthetic NS3<sub>1073-1081</sub> peptide. On day 2 of culture, 10% Rat-T-Stim (Collaborative Biomedical Products, Bedford, Massachusetts, USA) was added. On day 7, a standard  $^{51}\text{Cr}$  release assay was performed using NS3<sub>1073-1081</sub>-peptide-pulsed,  $^{51}\text{Cr}$ -labeled CIR-AAD cells as target cells, and a 40-fold excess of unlabeled CIR-AAD as previously described (36). Percent specific lysis was calculated as (experimental release – spontaneous release)  $\times 100$ /(maximum release – spontaneous release), in which spontaneous and maximum release reflect target cell lysis in the absence of effector cells and in the presence of 10% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA), respectively. Nonspecific lysis in the absence of peptide was less than 10% in all assays.

**Cytotoxicity assay using human T cell lines.** HCV NS3<sub>1073-1081</sub>-specific T cell lines were used to detect the NS3<sub>1073-1081</sub> epitope in the proteasome digests. For this purpose, HCV NS3<sub>1073-1081</sub>-specific, cytotoxic CD8<sup>+</sup> T cell lines were established by repetitive NS3<sub>1073-1081</sub> peptide stimulation (20) from PBMCs of HCV-recovered patients. HCV-recovered patients were followed in the Liver Diseases Section, NIDDK, NIH, and gave informed consent according to a protocol approved by the NIDDK Institutional Review Board. HCV-specific T cell lines were tested in a serum-free  $^{51}\text{Cr}$ -release assay (20) against TAP-deficient T2 target cells that had either been loaded with 1 M peptide overnight or incubated with 8-hour-proteasome digests for 12 hours in serum-free RPMI 1640 medium prior to labeling with 50 Ci  $^{51}\text{Cr}$ . Only NS3<sub>1073-1081</sub>-specific T cell lines with a sensitivity level

of at least 0.1 M peptide were tested against target cells loaded with the proteasomal digests.

**Statistical analysis.** Student's *t* test (two-tailed) was used to compare the frequency of NS3<sub>1073-1081</sub>-specific cells with their CTL activity in mice immunized with recombinant vaccinia virus expressing wild-type or mutant NS3, respectively. The Fisher exact probability test (two-tailed) was used to compare the frequency of the Y/F mutation in HLA-A2-positive and -negative patients.

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