

This is the peer reviewed version of the following article:

**HLA-B\*27 subtype specificity determines targeting and viral evolution of a hepatitis C virus-specific CD8+ T-cell epitope**

**Katja Nitschke, Alejandro Barriga, Julia Schmidt, Jörg Timm, Sergei Viazov, Thomas Kuntzen, Arthur Y. Kim, Georg M. Lauer, Todd M. Allen, Silvana Gaudieri, Andri Rauch, Christian M. Lange, Christoph Sarrazin, Thomas Eiermann, John Sidney, Alessandro Sette, Robert Thimme, Daniel López, and Christoph Neumann-Haefelin.**

**J Hepatol. 2014 Jan;60(1):22-9.**

which has been published in final form at

<https://doi.org/10.1016/J.JHEP.2013.08.009>

Published in final edited form as:

*J Hepatol.* 2014 January ; 60(1): . doi:10.1016/j.jhep.2013.08.009.

## HLA-B\*27 subtype specificity determines targeting and viral evolution of a hepatitis C virus-specific CD8+ T-cell epitope

Katja Nitschke<sup>1,2</sup>, Alejandro Barriga<sup>3</sup>, Julia Schmidt<sup>1</sup>, Jörg Timm<sup>4</sup>, Sergei Viazov<sup>4</sup>, Thomas Kuntzen<sup>5</sup>, Arthur Y. Kim<sup>6</sup>, Georg M. Lauer<sup>6</sup>, Todd M. Allen<sup>5</sup>, Silvana Gaudieri<sup>7,8</sup>, Andri Rauch<sup>9</sup>, Christian M. Lange<sup>10</sup>, Christoph Sarrazin<sup>10</sup>, Thomas Eiermann<sup>11</sup>, John Sidney<sup>12</sup>, Alessandro Sette<sup>12</sup>, Robert Thimme<sup>1</sup>, Daniel López<sup>3</sup>, and Christoph Neumann-Haefelin<sup>1</sup>

<sup>1</sup>Department of Medicine II, University of Freiburg, Freiburg, Germany <sup>2</sup>Faculty of Biology, University of Freiburg, Freiburg, Germany <sup>3</sup>Instituto de Salud Carlos III, Majadahonda, Madrid, Spain <sup>4</sup>Institute of Virology, University Hospital, University of Duisburg-Essen, Essen, Germany <sup>5</sup>Ragon Institute of MGH, MIT and Harvard, Charlestown, MA, USA <sup>6</sup>Division of Infectious Diseases, MGH, Boston, MA, USA <sup>7</sup>School of Anatomy, Physiology and Human Biology, University of Western Australia, Perth Australia <sup>8</sup>Institute of Immunology and Infectious Disease, Murdoch University, Perth, Western Australia, Australia <sup>9</sup>Department of Infectious Diseases, Bern University Hospital and University of Bern, Switzerland <sup>10</sup>Department of Medicine I, J. W. Goethe University Hospital, Frankfurt, Germany <sup>11</sup>Transfusion Medicine, HLA-Laboratory, University Hospital Hamburg-Eppendorf, Hamburg, Germany <sup>12</sup>La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA

### Abstract

**Background & Aims**—HLA-B\*27 is associated with spontaneous HCV genotype 1 clearance. HLA-B\*27-restricted CD8+ T-cells target three NS5B epitopes. Two of these epitopes are dominantly targeted in the majority of HLA-B\*27+ patients. In chronic infection, viral escape occurs consistently in these two epitopes. The third epitope (NS5B<sub>2820</sub>) was dominantly targeted in an acutely infected patient. This was in contrast, however, to the lack of recognition and viral escape in the large majority of HLA-B\*27+ patients. Here, we set out to determine the host factors contributing to selective targeting of this epitope.

**Methods**—Four-digit HLA class I typing and viral sequence analyses were performed in 78 HLA-B\*27+ patients with chronic HCV genotype 1 infection. CD8+ T-cell analyses were performed in a subset of patients. In addition, HLA/peptide affinity was compared for HLA-B\*27:02 and 05.

**Results**—The NS5B<sub>2820</sub> epitope is only restricted by the HLA-B\*27 subtype HLA-B\*27:02 (that is frequent in Mediterranean populations), but not by the prototype HLA-B\*27 subtype B\*27:05. Indeed, the epitope is very dominant in HLA-B\*27:02+ patients and is associated with viral escape mutations at the anchor position for HLA-binding in 12 out of 13 HLA-B\*27:02+ chronically infected patients.

© 2013 European Association of the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Corresponding author: Christoph Neumann-Haefelin, MD, Department of Medicine II, University of Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany, Tel and Fax: +49-761-270-33530, christoph.neumann-haefelin@uniklinik-freiburg.de.

Conflict of interest: The authors have no competing interests.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Conclusions**—The NS5B<sub>2820</sub> epitope is immunodominant in the context of HLA-B\*27:02, but is not restricted by other HLA-B\*27 subtypes. This finding suggests an important role of HLA subtypes in the restriction of HCV-specific CD8+ responses. With minor HLA subtypes covering up to 39% of specific populations, these findings may have important implications for the selection of epitopes for global vaccines.

### Keywords

Hepatitis C virus; CD8+ T-cell response; HLA subtypes; HLA-B\*27; vaccine design

### Introduction

After infection with hepatitis C virus (HCV), only a minority of patients can clear the virus spontaneously, while >70% of patients develop a chronic infection. Virus-specific CD8+ T-cells recognize HCV epitopes that are presented by HLA class I molecules on antigen-presenting cells as well as infected hepatocytes, and are essential effector cells in viral clearance[1]. Specific HLA class I types, such as HLA-B\*27 and B\*57, have a protective role in HCV infection, since they are associated with high rates of spontaneous viral clearance[2, 3]. We could link the protective effect of HLA-B\*27 to dominant HLA-B\*27 restricted epitopes located in the RNA-dependent RNA polymerase of HCV, the NS5B protein[4]. Indeed, one of these epitopes, NS5B<sub>2841</sub> (amino acid sequence ARMILMTHF), is very immunodominant, since it is targeted in nearly all HLA-B\*27+ patients with acute or resolved infection. Nearly all HLA-B\*27+ patients with chronic infection display multiple viral escape mutations in this epitope. Indeed, we could demonstrate that viral escape from this epitope requires several mutations in order to bypass recognition by the CD8+ T-cell response without deteriorating viral replication capacity. Most likely, this high barrier to viral escape explains why most HLA-B\*27+ patients successfully clear the virus in the acute phase of infection, before HCV can escape from the HLA-B\*27 restricted CD8+ T-cell response[5]. A second HLA-B\*27 restricted CD8+ T-cell epitope located in NS5B (NS5B<sub>2936</sub>, amino acid sequence GRAAICGKY) is subdominantly recognized and selects for viral escape mutations in approx. 50% of chronically HCV-infected HLA-B\*27+ patients[6].

Next to these two HLA-B\*27 restricted epitopes, we initially identified a third HLA-B\*27 restricted epitope located in NS5B (NS5B<sub>2820</sub>, amino acid sequence ARHTPVNSW) in the same acutely HCV-infected patient of Sicilian (southern Italian) origin[4]. This epitope was, however, not targeted in any patient from an Irish cohort of HLA-B\*27+ patients with recovered HCV infection, who were infected with a viral inoculum that contained the respective epitope sequence. Based on a more diverse expression of HLA-B\*27 subtypes in southern Italy (subtypes B\*27:05, B\*27:02, and B\*27:09) compared to Ireland (HLA-B\*27:05 only)[7], we hypothesized that the differential targeting of this HLA-B\*27 restricted CD8+ T-cell epitope may be due to a limited restriction by certain HLA-B\*27 subtypes. Indeed, here we demonstrate that the epitope NS5B<sub>2820</sub> is immunodominant exclusively in the context of HLA-B\*27:02. This HLA-B\*27 subtype is predominant (>50%) in the Middle East and Northern Africa but is only present in 5–10% of HLA-B\*27+ Euro-Caucasians[7]. People that express the ancestral and most common HLA-B\*27 subtype HLA-B\*27:05, however, do not recognize this epitope. These findings have important implications for vaccine design, since epitope targeting and immunodominance may be impacted not only by individual HLA types, but also by HLA subtypes.

## Patients and methods

### Patient cohorts

Patients with chronic HCV genotype 1 infection from four different cohorts (Table 1)[8–10] were tested for HLA-B\*27 expression by standard methods using molecular HLA typing or HLA serotyping procedures (n=1356). A subset of patients (n=165) was tested with an anti-HLA-B\*27 flow cytometry antibody (One lambda, Canoga Park, CA, USA) following the manufacturer's instructions. HLA-B\*27+ patients identified by this approach were subjected to 4-digit molecular HLA typing. Autologous viral sequences corresponding to the NS5B region that contains the NS5B<sub>2820</sub> epitope were determined as described previously[8–10]. PBMC from five HLA-B\*27:02+ patients with chronic HCV genotype 1 infection, PBMC from an HLA-B\*27:02+ patient with spontaneously resolved HCV infection (anti-HCV positive, HCV-RNA negative), PBMC from an HLA-B\*27:02+ patient of Sicilian origin with acute resolving HCV genotype 1a infection[4] (all presenting to the Freiburg University Medical Center), as well as PBMC from an HLA-B\*27:02+ patient with acute resolving HCV genotype 1a infection recruited at the Massachusetts General Hospital were isolated as described previously[4]. For control experiments, PBMC from an HLA-B\*27:05+ patient with acute resolving HCV genotype 1a infection, spontaneously resolved infection, and chronic HCV genotype 1a infection, respectively, recruited at the Freiburg University Medical Center were used. A cohort of six HLA-B\*27+ Irish women with resolved HCV genotype 1b infection has been described previously[4]. All patients gave informed consent; the study was performed in agreement with federal guidelines and the local ethics committees.

Generation of peptide-specific cell lines, intracellular interferon-gamma staining, and HLA-B\*27/peptide stability assays are described in the supplementary methods.

## Results

### The NS5B<sub>2820</sub> epitope is vigorously recognized in a Sicilian HLA-B\*27+ patient with acute HCV infection, but not in Irish and German HLA-B\*27+ patients

We previously identified three HLA-B\*27 restricted HCV NS5B-specific CD8+ T-cell epitopes in a Sicilian patient with acute HCV genotype 1a infection[4]. Two of these epitopes, NS5B<sub>2841</sub> (amino acid sequence ARMILMTHF) and NS5B<sub>2936</sub> (amino acid sequence GRAAICGKY) are frequently targeted in HLA-B\*27+ patients with resolved or chronic HCV genotype 1 infection. In addition, viral escape mutations are selected in these two epitopes in >90% and ~50%, respectively, of HLA-B\*27+ patients with chronic HCV genotype 1 infection[6, 11]. The third HLA-B\*27 restricted epitope, NS5B<sub>2820</sub> (amino acid sequence ARHTPVNSW), displayed the strongest interferon-gamma response in the acutely infected patient (Fig. 1A). Strikingly, however, we did not observe any CD8+ T-cell response against this epitope in 6 Irish HLA-B\*27+ patients with resolved HCV genotype 1b infection (Fig. 1B). Importantly, these patients were infected with a single source HCV strain harbouring the wild-type NS5B<sub>2820</sub> epitope sequence, indicating that sequence mismatches in the epitope region cannot explain the lack of recognition. In addition, we were also unable to detect any NS5B<sub>2820</sub>-specific CD8+ T-cell response in 8 HLA-B\*27+ German patients with chronic genotype 1b infection (data not shown). Sequence analyses revealed that 6 of the 8 HLA-B\*27+ patients had wild-type viral sequences in this epitope region, indicating the absence of substantial immune pressure (Fig. 1C).

Irish HLA-B\*27+ individuals primarily express the ancestral and prototype HLA-B\*27 subtype B\*27:05. This subtype is also predominant in German cohorts (reported at >90%). In contrast, in Mediterranean people such as Sicilians the subtype HLA-B\*27:02 is also

frequently expressed. We thus speculated that the expression of different HLA-B\*27 subtypes may impact the recognition of the NS5B<sub>2820</sub> epitope.

### **Epitope NS5B<sub>2820</sub> is restricted by the HLA-B\*27 subtype B\*27:02 and is not targeted in the context of the prototype subtype B\*27:05**

Interestingly, HLA-B\*27 subtype analysis showed that the Sicilian patient with acute HCV infection is positive for HLA-B\*27:02 and not the prototype B\*27:05. In order to test the hypothesis that the expression of HLA-B\*27:02 may be responsible for the targeting and unusual dominance of the NS5B<sub>2820</sub> epitope, we analyzed the CD8<sup>+</sup> T-cell response to the three HLA-B\*27-restricted NS5B epitopes in a second HLA-B\*27:02<sup>+</sup> patient with acute resolving HCV genotype 1a infection. Importantly, this patient showed a similar pattern of recognition of the three HLA-B\*27-restricted NS5B epitopes, with the NS5B<sub>2820</sub> epitope being the dominant target (supplementary Figure 1). Next, we tested the ability of HLA-B\*27:02 or B\*27:05 expressing antigen-presenting cells to present the NS5B<sub>2820</sub> peptide to an epitope-specific T-cell line derived from the Sicilian patient. When we loaded HLA-B\*27:02 expressing EBV-transformed B-cell lines with peptide and co-cultured these cells with an NS5B<sub>2820</sub>-specific T-cell line, we indeed observed a strong interferon-gamma response (Fig. 2A, upper panel). When we used HLA-B\*27:05 expressing EBV-transformed B-cell lines, however, we did not observe any interferon-gamma production (Fig. 2A, lower panel), even when loading B-cells with 10-fold peptide concentrations (data not shown). The same result was obtained when the B-cell lines were not externally loaded with the epitope peptide, but an endogenous antigen processing experiment was performed using HCV-expressing vaccinia constructs (Fig. 2A). Equivalent results were also obtained when NS5B<sub>2820</sub>-specific T-cell lines were used that had been generated from HLA-B\*27:02<sup>+</sup> patients with spontaneously resolved HCV infection or chronic HCV genotype 1a infection, respectively (supplementary Figure 2). These data clearly demonstrate that epitope NS5B<sub>2820</sub> is restricted by HLA-B\*27:02, but not by the HLA-B\*27 prototype B\*27:05.

We also stimulated PBMC from three HLA-B\*27:05<sup>+</sup> patients with acute resolving, spontaneously resolved, and chronic HCV genotype 1a infection, respectively, with the NS5B<sub>2820</sub> epitope peptide for two weeks. However, we were unable to detect epitope-specific CD8<sup>+</sup> T-cell responses even when using 10-fold peptide concentrations in cell culture and/or read-out procedures, further indicating that HLA-B\*27:05<sup>+</sup> patients do not target this epitope (data not shown).

HLA-B\*27:02 is the only HLA-B\*27 subtype that has been shown to accept tryptophan (W) at the C-terminus of antigen ligands[12]. The NS5B<sub>2820</sub> epitope has indeed a tryptophan at its C-terminus. We therefore hypothesized that the NS5B<sub>2820</sub> epitope peptide may bind specifically to HLA-B\*27:02 molecules, but not or only weakly to HLA-B\*27:05 molecules. HLA binding analyses indeed showed that the NS5B<sub>2820</sub> peptide has a high affinity for HLA-B\*27:02, while it displayed an inferior binding affinity to HLA-B\*27:05, when compared to other known HLA-B\*27 restricted viral CD8<sup>+</sup> T-cell epitopes, including the HCV-specific NS5B<sub>2841</sub> and NS5B<sub>2936</sub> epitopes, and also the HIV-specific gag p24 epitope KK10 (Fig. 2B). The relatively weak binding of NS5B<sub>2820</sub> to HLA-B\*27:05 was confirmed in a competition assay using radiolabeled standard peptide (Table 2). *In silico* modeling of the binding of the NS5B<sub>2820</sub> peptide in the HLA-B\*27 peptide binding groove[13, 14] indicated that the C-terminal tryptophan of NS5B<sub>2820</sub> may indeed better embed in the F pocket of HLA-B\*27:02 compared to the F pocket of HLA-B\*27:05 (Fig. 3). Of note, in addition to the relatively small differences observed by *in silico* modeling, additional conformational changes may further impact NS5B<sub>2820</sub> binding to the different HLA-B\*27 subtype molecules[15]. Taken together, these *in vitro* and *in silico* results further

support the conclusion that the NS5B<sub>2820</sub> epitope is only presented by HLA-B\*27:02 and not by HLA-B\*27:05.

### Escape mutations in the NS5B<sub>2820</sub> epitope are reproducibly selected in HLA-B\*27:02+ patients

Since we demonstrated the epitope NS5B<sub>2820</sub> to be specifically restricted by the subtype B\*27:02, we aimed to analyze this epitope in a larger cohort of HLA-B\*27:02+ patients. In order to include a sufficient number of HLA-B\*27:02+ patients, we combined data from four large cohorts of patients with chronic HCV genotype 1 infection, including a total of 1521 patients. 91 (6.0%) of these patients were HLA-B\*27+ (Table 1). In a total of 78 HLA-B\*27+ patients, 4-digit typing of HLA-B\*27 as well as viral sequence data for the NS5B region corresponding to the NS5B<sub>2820</sub> epitope could be obtained. Importantly, in 12 out of 13 (92.3%) HLA-B\*27:02+ patients, we observed a sequence variation at amino acid position 2 of the epitope, with a substitution of arginine (R) by lysine (K) (Table 3). In contrast, patients that expressed the HLA-B\*27 prototype B\*27:05 rarely displayed sequence variations compared to the consensus sequence (6/59 patients, 10.2%;  $P < 0.0001$ , Fisher's exact test). Only a single HLA-B\*27:05+ patient (1.7%) displayed a mutation at position 2 of the epitope, suggesting that the few mutations occurring in HLA-B\*27:05+ patients were not driven by NS5B<sub>2820</sub>-specific CD8+ T-cells. Patients expressing rare HLA-B\*27 subtypes such as B\*27:03, B\*27:06, B\*27:10, and B\*27:14 showed similar results compared to HLA-B\*27:05+ patients, with only 1 of 6 patients displaying a variant viral sequence.

Our findings indicate that NS5B<sub>2820</sub>-specific CD8+ T-cells are strong drivers of viral evolution in HLA-B\*27:02+ patients, resulting in the viral escape mutation R2821K at amino acid position 2 of the epitope. Arginine (R) at this position functions as the main HLA-B\*27 binding anchor, thus, mutational escape at this position is likely to have a strong impact on epitope presentation. Indeed, the R2821K mutant peptide displayed only marginal binding to the HLA-B\*27:02 molecule as compared to the wild-type peptide (Fig. 4A). We next tested recognition of the consensus as well as variant peptide by epitope-specific cell lines generated from the patient with acute infection. As shown in Fig. 4B (upper panel), the R2821K mutation completely abolished recognition of the epitope peptide, demonstrating that this viral variation does indeed represent a viral escape mutation.

We also tested PBMC from five HLA-B\*27:02+ patients with chronic HCV genotype 1 infection and PBMC from one HLA-B\*27:02+ patient with spontaneously resolved HCV infection for NS5B<sub>2820</sub>-specific CD8+ T-cell responses after two weeks of peptide-specific cell culture. In one out of the five chronically infected patients as well as in the patient with resolved infection, we were successful in generating epitope-specific CD8+ T-cell lines with strong interferon-gamma production in response to the consensus, but not the R2821K variant peptide (Fig. 4B, middle and lower panel). It is important to note, that the frequency of epitope-specific responses observed here (one response in five chronically infected patients) is similar to that observed for other immunodominant HCV-specific CD8+ T-cell epitopes in the setting of chronic infection[16]. We further confirmed that mutation R2821K represents an escape variant by performing peptide titration assays for the NS5B<sub>2820</sub>-specific T-cell lines (Fig. 4C). Of note, the partial cross-recognition of the mutant peptide in the chronically infected patient in this assay (Fig. 4C) in contrast to the lack of cross-recognition after loading of the mutant peptide on HLA-B\*27:02+ B-cells with subsequent washing of the B-cells prior to co-culture with the epitope-specific T-cell line (Fig. 4B) indicates that the variant peptide binds to HLA-B\*27:02 with low affinity, resulting in a high drop-off rate.

It has been described that certain viral mutant peptides may have antagonistic capacities and compete with their respective wild-type peptide for HLA and/or TCR binding[17]. Thus, we performed competition assays titrating increasing doses of the mutant peptide in addition to the wild-type peptide, however, we observed no antagonistic effect (Fig. 4D).

In summary, we clearly show that the epitope NS5B<sub>2820</sub> is specifically restricted by HLA-B\*27:02, but not by the prototype HLA-B\*27 subtype B\*27:05. In the context of HLA-B\*27:02, viral escape mutations are selected in the vast majority of patients. These results suggest that HLA class I subtypes have an important impact in directing the HCV-specific CD8<sup>+</sup> T-cell response.

## Discussion

We have previously identified three HLA-B\*27 restricted CD8<sup>+</sup> T-cell epitopes located in the HCV NS5B protein[4]. Two of these epitopes, NS5B<sub>2841</sub> and NS5B<sub>2936</sub>, were frequently targeted in HLA-B\*27<sup>+</sup> patients with HCV genotype 1 infection and also reproducibly induced viral escape. In the current study, we analyzed a third HLA-B\*27 restricted epitope in NS5B. This epitope seemed to be a minor CD8<sup>+</sup> T-cell target, since we could not detect CD8<sup>+</sup> T-cell responses against this epitope in any of 14 HLA-B\*27<sup>+</sup> patients with resolved or chronic HCV infection. This finding was somewhat in discrepancy with the very strong epitope-specific CD8<sup>+</sup> T-cell response observed in a patient with acute HCV infection. HCV genotype or subtype-diversity could not explain this finding, since the epitope is conserved in geno-/subtypes 1a, 1b, and also 3a (supplementary table 1). When we analyzed the reason for these discrepant observations, we discovered that the epitope NS5B<sub>2820</sub> is only presented by the HLA-B\*27 subtype B\*27:02, but not by other HLA-B\*27 subtypes, including the prototype HLA-B\*27:05. The HLA-B\*27:02 subtype-specificity of this epitope may be largely mediated by a superior binding of the epitope peptide to HLA-B\*27:02 and an only weak binding to HLA-B\*27:05. However, other mechanisms such as naïve precursor frequencies may also have an additional impact[18].

We found that NS5B<sub>2820</sub> is a dominant epitope in HLA-B\*27:02 patients, since the vast majority (12 of 13) of HLA-B\*27:02<sup>+</sup> patients with chronic HCV infection displayed a viral escape mutation at the main HLA binding anchor of the epitope. Since this epitope is only restricted by the relatively rare subtype HLA-B\*27:02, but not by the common 'ancestral' B\*27:05, it is by mistake considered not to be immunodominant if HLA-B\*27 subtypes are not taken into account. Thus, host genetic diversity at the HLA-B\*27 subtype level has an important impact on epitope recognition and may influence protective immune responses in a similar way as has been reported for HCV genotypes that do or do not contain specific protective epitopes[11]. Of note, recognition as well as viral escape in the HLA-B\*27-restricted epitopes NS5B<sub>2841</sub> and NS5B<sub>2936</sub> do not differ between HLA-B\*27:05 and HLA-B\*27:02 (data not shown), indicating that a differential targeting of epitopes by these two HLA-B\*27 subtypes is not mandatory.

Due to a lack of large studies analyzing the impact of HLA class I alleles at high resolution (4-digit HLA subtypes), it is not clear if HLA-B\*27:02 has a different (stronger or weaker) protective role in HCV or HIV infection compared to HLA-B\*27:05. In this context it is important to point out, however, that the protective effect of HLA-B\*27 in HCV infection has been demonstrated most clearly in an Irish cohort[3]. The Irish population nearly exclusively expresses the HLA-B\*27 subtype B\*27:05, and we previously linked the protective effect of HLA-B\*27 in that cohort to the dominant targeting of the NS5B<sub>2841</sub> epitope[4]. Thus, the HLA-B\*27:02-specific NS5B<sub>2820</sub> epitope is most likely not involved in conferring protection in the Irish cohort. Of note, differential effects on the clinical course of infection have been described for other HLA subtypes. For example, the HLA subtype

B\*35:01, but not B\*35:02/03 is hazardous in HIV infection. Even more strikingly, B\*58:01 is protective, but B\*58:02 is detrimental in HIV infection[19, 20]. Similarly, HLA-B\*38 is hazardous in HCV infection, while the highly related B\*39 is protective in HCV infection[21]. Thus, very small differences in closely related HLA class I molecules can have relevant effects on both, CD8+ T-cell recognition as well as immune control.

Our finding may have important implications for HCV vaccine design. Indeed, identification of HCV-specific CD8+ T-cell epitopes has been mainly performed in North-American and European Caucasians. HLA class I subtypes were not considered in most of these studies, and thus little is known about the exact restriction of most HCV-specific CD8+ T-cell epitopes. HLA prototype subtypes are defined as those HLA subtypes most prevalent in a certain population. For example, HLA-A\*02:01 is present in >90% of HLA-A\*02+ Euro-Caucasians, while all other HLA-A\*02 subtypes are rare in this population. Thus, A\*02:01 is the HLA-A\*02 prototype. These prototypes do cover 96% and 87% of all HLA-A and HLA-B subtypes expressed e.g. in US Caucasians[22]. When these prototypes defined in US Caucasians are used to study ethnically distinct population, e.g. African-Americans, Asians or Hispanics living in the US, coverage rates drop to 64–80% for HLA-A subtypes and 61–63% for HLA-B subtypes. For example, HLA-A\*02:01 is expressed by less than 50% of HLA-A\*02+ individuals of Afro-American or Asian origin in the US. In consequence, analysis of ethnically mixed populations using e.g. only HLA-A\*02:01 and HLA-B\*27:05 restricted CD8+ epitopes may not be appropriate.

Since our patient cohort consisted mainly (>90%) of patients of Caucasian ethnicity, we could not perform a comprehensive analysis of the impact of HLA subtypes on HCV-specific CD8+ T-cell responses in HCV infection. We performed a preliminary analysis, however, in 287 patients with HCV genotype 1a infection, known 4-digit HLA types and known autologous full-genome HCV sequences[9]. In this cohort, > 5 patients expressed non-prototype alleles of the HLA-B types B\*14, B\*15, B\*35, B\*40, and B\*44, respectively. Of these HLA-B types, only B\*15, B\*35 and B\*40 displayed HLA associations within known CD8+ T-cell epitopes. Interestingly, in four of the five epitopes, we found evidence for HLA subtype-specific selection of viral escape mutations (supplementary Fig. 3), indicating that indeed subtypes of several HLA-B alleles may play an important role in restriction of HCV-specific CD8+ T-cell responses.

While these findings clearly need to be confirmed in additional studies performed in cohorts with differential ethnical backgrounds, they indicate that vaccine candidates that have been designed considering HLA class I prototypes only may exclude protective CD8+ T-cell epitopes restricted by minor HLA subtypes. In this scenario, it is important to note that rare HLA types and subtypes may predominantly contribute to viral control[23]. As a consequence, vaccine studies should aim at a broad coverage of potential CD8+ epitopes and also include comprehensive analyses using e.g. overlapping peptides in their read-outs in order to avoid a bias towards prototype HLA subtypes.

In conclusion, we demonstrate here that HLA class I subtypes may have an important role in restriction of HCV-specific CD8+ T-cell epitopes. This is highlighted in our study of an HLA-B\*27 restricted CD8+ T-cell epitope that is immunodominant in the background of HLA-B\*27:02, but not presented by other HLA-B\*27 subtypes, including the prototype HLA-B\*27:05.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Financial support: CNH was supported by the Deutsche Forschungsgemeinschaft (DFG; Emmy Noether Program, NE 1567/1-1). CNH and RT were supported by the European Union (EFRE Interreg IV Oberrhein, project A30). SV was supported by the German Ministry of Education and Research (BMBF, grant no. 01 KI 1008E). DL was supported by the Ministerio de Ciencia e Innovación, Spain. This project was funded, in part, by Federal funds from the National Institute of Allergy and Infectious Diseases (NIAID), grants R01-AI067926 (TMA) and U19 AI082630 (TMA and GML), and the Swiss HIV Cohort Study, supported by the Swiss National Science Foundation (grant #33CS30\_134277).

We would like to thank all patients who donated blood for this study. We also appreciate the help of all physicians and study nurses who included patients into the cohort.

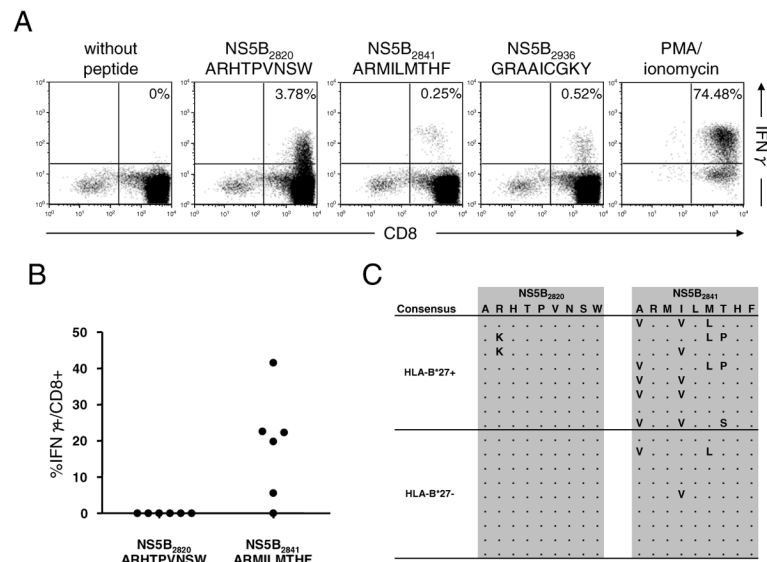
## List of abbreviations in the order of appearance

<b>HLA</b>	human leukocyte antigen
<b>HCV</b>	hepatitis C virus
<b>NS5B</b>	non-structural protein 5B
<b>PBMC</b>	peripheral blood mononuclear cell
<b>EBV</b>	Epstein Barr virus
<b>B-LCL</b>	B-lymphoblastoid cell lines

## References

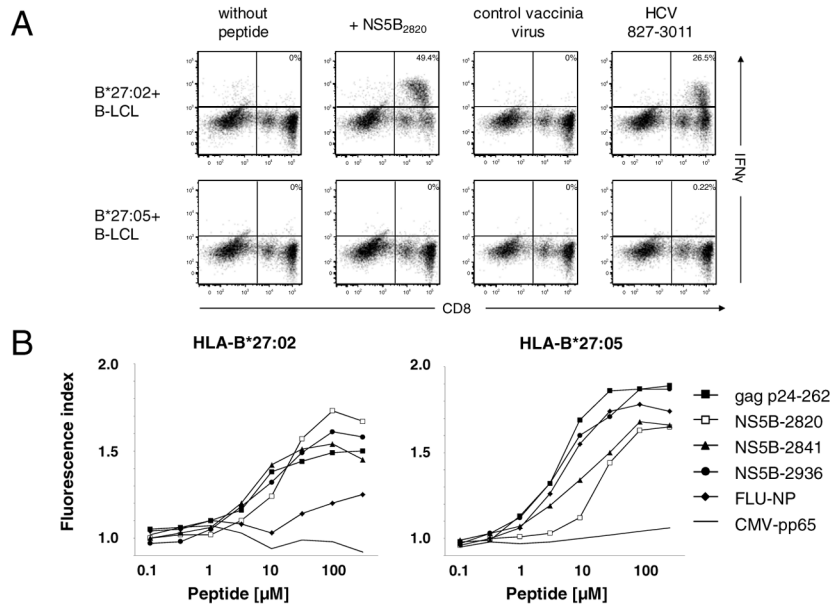
- Walker CM. Adaptive immunity to the hepatitis C virus. *Adv Virus Res.* 2010; 78:43–86. [PubMed: 21040831]
- Kuniholm MH, Kovacs A, Gao X, Xue X, Marti D, Thio CL, et al. Specific human leukocyte antigen class I and II alleles associated with hepatitis C virus viremia. *Hepatology.* 2010; 51:1514–1522. [PubMed: 20169624]
- McKiernan SM, Hagan R, Curry M, McDonald GS, Kelly A, Nolan N, 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. [PubMed: 15239092]
- Neumann-Haefelin C, McKiernan S, Ward S, Viazov S, Spangenberg HC, Killinger T, et al. Dominant influence of an HLA-B27 restricted CD8+ T cell response in mediating HCV clearance and evolution. *Hepatology.* 2006; 43:563–572. [PubMed: 16496339]
- Dazert E, Neumann-Haefelin C, Bressanelli S, Fitzmaurice K, Kort J, Timm J, et al. Loss of viral fitness and cross-recognition by CD8+ T cells limit HCV escape from a protective HLA-B27-restricted human immune response. *J Clin Invest.* 2009; 119:376–386. [PubMed: 19139562]
- Neumann-Haefelin C, Oniangue-Ndza C, Kuntzen T, Schmidt J, Nitschke K, Sidney J, et al. Human leukocyte antigen B27 selects for rare escape mutations that significantly impair hepatitis C virus replication and require compensatory mutations. *Hepatology.* 2011; 54:1157–1166. [PubMed: 22006856]
- Blanco-Gelaz MA, Lopez-Vazquez A, Garcia-Fernandez S, Martinez-Borra J, Gonzalez S, Lopez-Larrea C. Genetic variability, molecular evolution, and geographic diversity of HLA-B27. *Hum Immunol.* 2001; 62:1042–1050. [PubMed: 11543905]
- Gaudieri S, Rauch A, Pfafferoth K, Barnes E, Cheng W, McCaughan G, et al. Hepatitis C virus drug resistance and immune-driven adaptations: relevance to new antiviral therapy. *Hepatology.* 2009; 49:1069–1082. [PubMed: 19263475]
- Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology.* 2008; 48:1769–1778. [PubMed: 19026009]

10. Lange CM, Roomp K, Dragan A, Nattermann J, Michalk M, Spengler U, et al. HLA class I allele associations with HCV genetic variants in patients with chronic HCV genotypes 1a or 1b infection. *J Hepatol.* 2010; 53:1022–1028. [PubMed: 20800922]
11. Neumann-Haefelin C, Timm J, Schmidt J, Kersting N, Fitzmaurice K, Oniangue-Ndza C, et al. Protective effect of human leukocyte antigen B27 in hepatitis C virus infection requires the presence of a genotype-specific immunodominant CD8+ T-cell epitope. *Hepatology.* 2010; 51:54–62. [PubMed: 20034048]
12. Rotzschke O, Falk K, Stevanovic S, Gnau V, Jung G, Rammensee HG. Dominant aromatic/aliphatic C-terminal anchor in HLA-B\*2702 and B\*2705 peptide motifs. *Immunogenetics.* 1994; 39:74–77. [PubMed: 8225441]
13. Kumar N, Mohanty D. MODPROPEP: a program for knowledge-based modeling of protein-peptide complexes. *Nucleic acids research.* 2007; 35:W549–555. [PubMed: 17478500]
14. Sundaramurthi JC, Ramanathan VD, Hanna LE. HLA-B\*27:05-specific cytotoxic T lymphocyte epitopes in Indian HIV type 1C. *AIDS research and human retroviruses.* 2013; 29:47–53. [PubMed: 22924625]
15. Infantes S, Lorente E, Barnea E, Beer I, Barriga A, Lasala F, et al. Natural HLA-B\*2705 protein ligands with glutamine as anchor motif: implications for HLA-B27 association with spondyloarthropathy. *J Biol Chem.* 2013; 288:10882–10889. [PubMed: 23430249]
16. Lauer GM, Barnes E, Lucas M, Timm J, Ouchi K, Kim AY, et al. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology.* 2004; 127:924–936. [PubMed: 15362047]
17. Bertoletti A, Sette A, Chisari FV, Penna A, Levrero M, De Carli M, et al. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature.* 1994; 369:407–410. [PubMed: 8196768]
18. Schmidt J, Neumann-Haefelin C, Altay T, Gostick E, Price DA, 3 VL, Hubert E, Blum1, Robert Thimme1. Immunodominance of HLA-A2 restricted hepatitis C virus-specific CD8+ T cell responses is linked to naïve precursor frequency. *J Virol.* 2011; 85:5232–5236. [PubMed: 21367907]
19. Gao X, Nelson GW, Karacki P, Martin MP, Phair J, Kaslow R, et al. Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS. *N Engl J Med.* 2001; 344:1668–1675. [PubMed: 11386265]
20. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, Chetty S, et al. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature.* 2004; 432:769–775. [PubMed: 15592417]
21. Hrabec P, Kuiken C, Yusim K. Evidence for human leukocyte antigen heterozygote advantage against hepatitis C virus infection. *Hepatology.* 2007; 46:1713–1721. [PubMed: 17935228]
22. Maiers M, Gragert L, Klitz W. High-resolution HLA alleles and haplotypes in the United States population. *Hum Immunol.* 2007; 68:779–788. [PubMed: 17869653]
23. Trachtenberg E, Korber B, Sollars C, Kepler TB, Hrabec PT, Hayes E, et al. Advantage of rare HLA supertype in HIV disease progression. *Nat Med.* 2003; 9:928–935. [PubMed: 12819779]



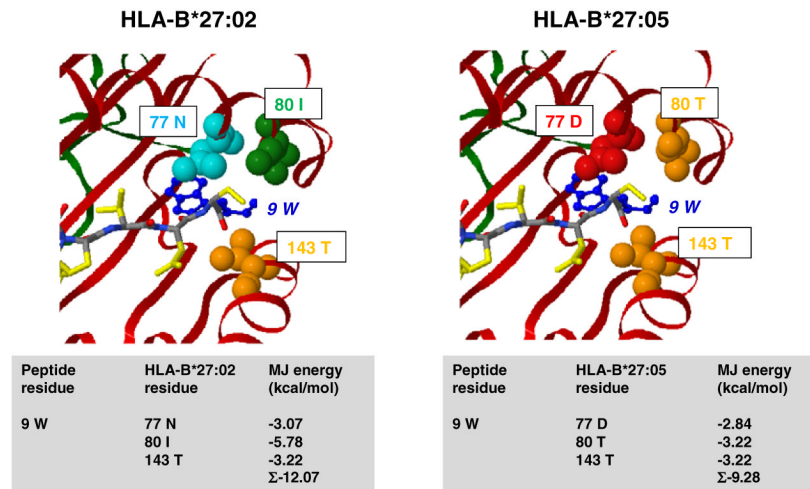
**Figure 1. HLA-B\*27 restricted epitope NS5B<sub>2820</sub> displays a vigorous CD8+ T-cell response in an HLA-B\*27+ patient with acute HCV infection, but is not targeted in HLA-B\*27+ patients with resolved or chronic infection**

**A.** CD8+ T-cells from an HLA-B\*27+ patient of Sicilian origin with acute resolving HCV genotype 1a infection were tested for interferon-gamma production in response to three HLA-B\*27 restricted epitopes located in NS5B. A sample without peptide stimulation and a sample stimulated with PMA and ionomycin were included as negative and positive controls, respectively. The percentage of interferon-gamma producing CD8+ T-cells is indicated. **B.** PBMC from 6 Irish individuals with spontaneously resolved HCV genotype 1b infection were stimulated for 14 days in the presence of the NS5B<sub>2820</sub> or NS5B<sub>2841</sub> epitope peptides, respectively, and tested for interferon-gamma production in response to the respective peptide. **C.** Autologous viral sequences corresponding to the NS5B<sub>2820</sub> epitope have been determined from 8 HLA-B\*27+ patients as well as 9 HLA-B\*27-control patients with chronic HCV genotype 1b infection. Each line represents the autologous sequence from one patient; a dot indicates identity to consensus. Previously published sequences corresponding to the NS5B<sub>2841</sub> epitope are shown for comparison[4].



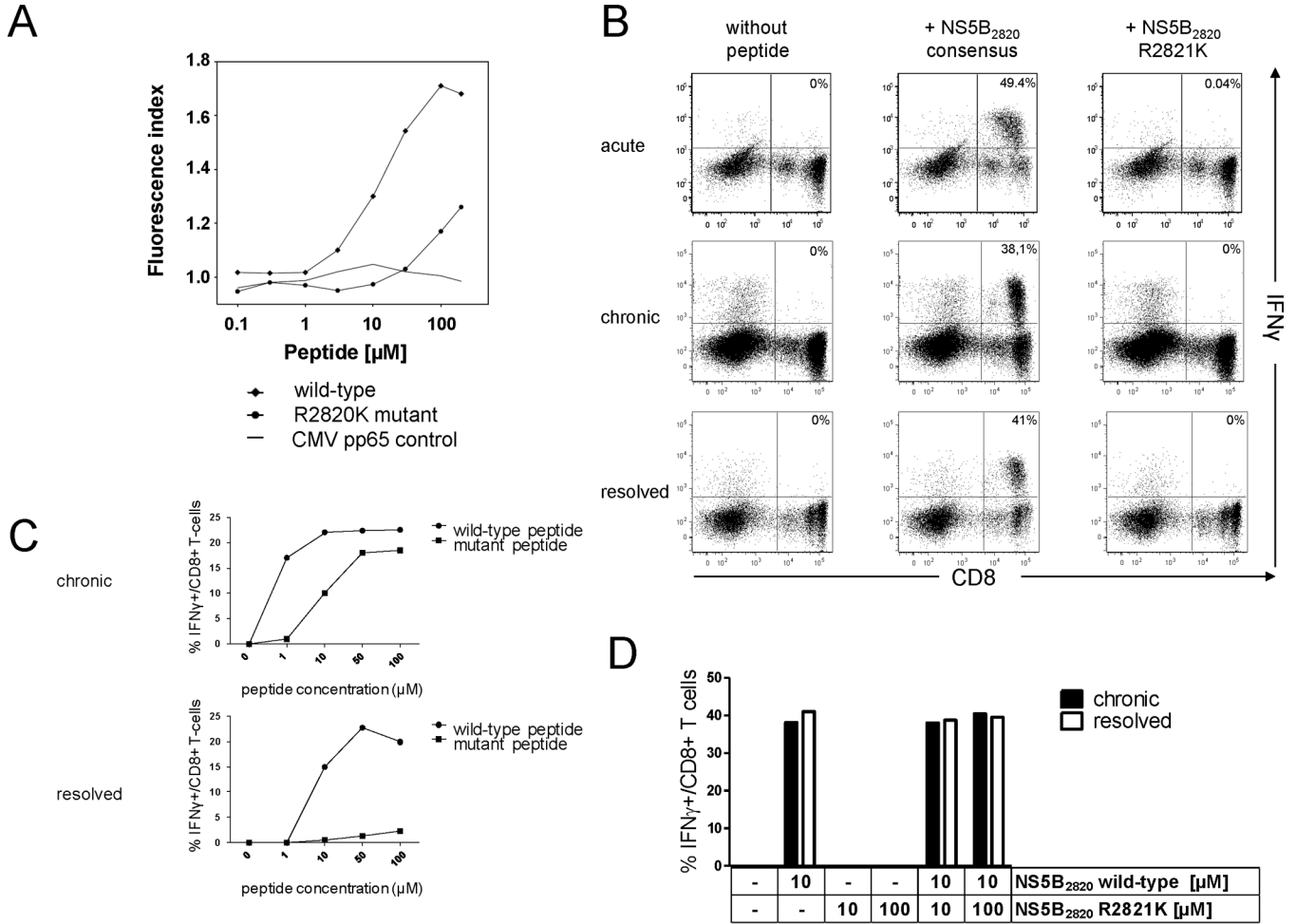
**Figure 2. Epitope NS5B<sub>2820</sub> is restricted by subtype HLA-B\*27:02 only, and is not targeted in the context of the HLA-B\*27 ‘prototype’ HLA-B\*27:05**

**A.** An NS5B<sub>2820</sub> epitope-specific CD8<sup>+</sup> T-cell line was generated from the HLA-B\*27<sup>+</sup> patient with acute resolving HCV genotype 1a infection by two weeks of peptide stimulation. The T-cell line was then co-cultured with EBV-transformed B-cells that expressed HLA-B\*27:02 or HLA-B\*27:05 and were or were not loaded with peptide NS5B<sub>2820</sub>. As read-out, intracellular interferon-gamma staining was performed (two panels on the left). Alternatively to peptide loading, the B-cell lines were infected with vaccinia viruses that did or did not express HCV amino acids 827–3011, including the NS5B protein (two panels on the right). **B.** HLA-B\*27:02 and B\*27:05 binding affinities were assessed for the NS5B<sub>2820</sub> as well as other known HLA-B\*27-restricted viral CD8<sup>+</sup> T-cell epitopes. Synthetic peptides corresponding to HIV gag p24<sub>262</sub> (KK10), NS5B<sub>2820</sub>, NS5B<sub>2841</sub>, NS5B<sub>2936</sub>, and Flu NP as well as CMV pp65 as negative control were titrated bound to HLA-B\*27:02 and B\*27:05, respectively, on RMA-S TAP-deficient cells, and stabilization was measured by flow cytometry. Data are means of 3–4 independent experiments.



**Figure 3. NS5B<sub>2820</sub> epitope peptide modeled into the binding groove of the HLA-B\*27:02 molecule (left) and the HLA-B\*27:05 molecule (right)**

Miyazawa and Jernigan (MJ) energy levels were calculated for the interaction between the C-terminal tryptophan of the epitope peptide and its modeled interaction partners in the F pocket of the HLA molecules. Analyses were performed using the MODPROPEP web server and the HLA-B\*27:05 (PDB ID: 1OGT) structure. A negative MJ energy favours stable binding.



**Figure 4. The R2821K mutation mediates viral escape from the NS5B<sub>2820</sub> epitope-specific CD8+ T-cell response**

**A.** HLA-B\*27:02 binding of the wild-type and R2821K mutant NS5B<sub>2820</sub> epitope peptides were compared in an assay equivalent to Fig. 2B. **B.** NS5B<sub>2820</sub> epitope-specific T-cell lines generated from HLA-B\*27:02+ patients with acute (upper panel), chronic (middle panel), or resolved (lower panel) HCV genotype 1a infection were co-cultured with an HLA-B\*27:02+ EBV-transformed B-cell line that has been loaded with the consensus NS5B<sub>2820</sub> epitope peptide or the variant epitope peptide containing the R2821K mutation. Intracellular interferon-gamma staining was performed as read-out. **C.** NS5B<sub>2820</sub>-specific T-cell lines from an HLA-B\*27:02+ patient with chronic HCV genotype 1a infection (upper panel) or resolved HCV infection (lower panel) were stimulated with the NS5B<sub>2820</sub> wild-type epitope peptide or the R2821K mutant peptide in increasing concentrations as indicated. **D.** The R2821K variant peptide does not act as an antagonist for the wild-type peptide. NS5B<sub>2820</sub>-specific T-cell lines from an HLA-B\*27:02+ patient with chronic HCV genotype 1a infection (filled bars) and spontaneously resolved HCV infection (white bars), respectively, were co-cultured with HLA-B\*27:02+ B-cell lines that had been loaded with wild-type or variant peptide in different concentrations as indicated.

**Table 1**  
 HLA-B\*27 frequency and subtype distribution in patients with chronic HCV genotype 1 infection

Cohort	Country of recruitment <sup>a</sup>	n patients (viral subtype distribution) <sup>b</sup>	n HLA-B*27+	% HLA-B*27+	n HLA-B*27 subtypes and viral sequence available			
					total	B*27:02	B*27:05	others
Freiburg	D	569 (1a: 179; 1b: 308; Ins: 82)	35	6.2	31	6	24	1 <sup>c</sup>
Boston	USA, D, CH	557 (1a: 405; 1b: 152)	27	4.8	24	3	16	5 <sup>d</sup>
Perth	AUS, CH, GB	236 (1a: 185; 1b: 51)	13	5.5	13	2	11	0
Frankfurt	D	159 (1a: 69; 1b: 87; Ins: 3)	16	10.1	10	2	8	0
Total		1521	91	6.0	78	13	59	6

<sup>a</sup>Country code: D: Germany; CH: Switzerland; AUS: Australia; GB: Great Britain

<sup>b</sup>ns: subtype not determined or mixed infection

<sup>c</sup>B\*27:14

<sup>d</sup>2 x B\*27:03, 2 x B\*27:06; B\*27:10

**Table 2**

HLA-B\*27:05 binding affinity determined in a competition assay using radiolabeled standard peptide

Peptide name	Epitope Sequence	HLA-B*27:05 (IC <sub>50</sub> nM)
NS5B-2820	ARHTPVNSW	338
NS5B-2841	ARMILMTHF	1.1
NS5B-2936	GRAAICGKY	1.0
FLU-NP	SRYWAIRTR	0.44
HIV gag p24–262	KRWIILGLNK	0.22

**Table 3**

Autologous viral sequences corresponding to the NS5B<sub>2820</sub> epitope

Consensus	A	R	H	T	P	V	N	S	W	n patients	% patients
B*27:02+	.	K	.	.	.	.	.	.	.	12/13	92.3%
B*27:05+	.	.	.	.	.	.	.	.	.	1/13	7.7%
	.	.	.	.	.	.	.	.	.	53/59	89.8%
	.	.	.	.	I	.	.	.	.	3/59	5.1%
	.	R	.	.	I	.	.	.	.	2/59	3.4%
	.	K	.	.	.	.	.	.	.	1/59	1.7%
B*27+, other subtypes	.	.	.	.	.	.	.	.	.	5/6	83.3%
	.	.	.	.	I	.	.	.	.	1/6	16.7%