Abschlussbericht Teilprojekt 16.2

Projekttitel: Immundominanz von CD8 T-Zellepitopen bei der akuten und chronischen HCV-Infektion und Rolle von viralem Escape im Verlauf der Infektion

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1. Summary

Virus-specific CD8+ T cell responses play an important role in the outcome of hepatitis C virus (HCV) infection. However, the mechanisms of viral clearance versus persistence have not been well understood so far. It was the aim of this study to identify protective CD8+ T cell epitopes and to clarify the role of viral evolution in the outcome of infection. We identified an imunodominant HLA-A26-restricted epitope that was targeted by 3 out of 3 HLA-A26 positive patients. Interestingly, all three patients spontaneously cleared viremia. Viral sequencing revealed that this epitope is highly conserved even in the presence of HLA A26, raising the possibility that it may be protective due to its high grade of conservation and that viral persistence requires mechanisms other than viral escape in the context of this epitope. In a second project the extent of escape from intrahepatic T cells was determined in a cohort of 20 chronically HCV infected patients. In >50% of CD8 epitopes targeted by intrahepatic T cells the autologous viral sequence differs from the consensus sequence and T cell recognition of the variant is substantially compromised consistent with escape. This indicates that mutational escape can be reproducible and widespread. Finally we demonstrated an example for which escape mutations in an immunodominant epitope can accumulate in a population infected with HCV and dissected out the evolutionary forces contributing to this observation. This analysis revealed multiple factors shaping the evolution in an immunodominant CD8 epitope in which the reproducibility of the immune response, the frequency of the restricting HLA-allele in a population and the fitness costs associated with escape mutations play a key role. These results are fundamental to the future design of therapeutic vaccines.

2. Original aims of the project

It was the original overall aim of this study to determine the immunological basis for the association of 'protective' HLA class I alleles and HCV clearance and to identify immunodominant epitopes in HCV infection and the role of viral evolution. We therefore proposed the following specific aims:

1.) Identification of immunodominant CD8 epitopes in patients with acute HCV infection.
2.) Comparison with the immunodominance of CD8 T cells in subjects with chronic and resolved HCV infection.
3.) Characterization of the impact of specific CD8 T cells on viral evolution and its contribution to viral persistence.

3. Scientific results

Growing evidence suggests an important role of the adaptive T cell response for control of acute hepatitis C virus (HCV) infection (1-3). The mechanisms by which HCV establishes persistent infection are not fully understood. T cell failure may be caused by selection of viral escape mutations within targeted MHC-class I restricted HCV epitopes (4-8). However, the determinants of viral escape as well as its relative contribution to T cell failure and HCV persistence are not well characterized to date.

4. Lack of escape in an immunodominant HLA-A26 restricted CD8+ epitope identified during acute HCV infection

In the previous progress reports we highlighted an immunodominant HLA-A26 restricted epitope that was targeted by 3/3 (100%) HLA-A26+ subjects with acute infection. Interestingly, all three subjects spontaneously resolved viremia. Longitudinal analysis of viral sequences of the targeted region in one subject revealed that no mutations were selected during the acute phase of infection. This is in contrast to other targeted immunodominant epitopes for which selection of escape mutations during acute infection has been well documented and has been suggested as one important mechanism for the failure of the virus-specific immune response (4, 5, 7, 8). However, a longitudinal study described lack of

mutational escape in a subject with acute infection and with spontaneous resolution of viremia suggesting this as a potential correlate for control (8). To determine if viral persistence is associated with mutational escape in the context of this immunodominant HLA-A26 restricted CD8 epitope we next studied the autologous viral sequences corresponding to the epitope in HLA-A26+ patients with chronic HCV infection. Strikingly, no viral sequence variations were detected within this epitope in nine HLA-A26+ patients studied, indicating that viral escape had not taken place. Of note, two patients with CD8+ T cell responses against this epitope were included. Our findings support previous observations suggesting that high functional constraints may be present in this region (9, 10). These constraints likely prevent mutational escape in the targeted HLA-A26 epitope and illustrate the limits of viral evolution in the presence of selection pressure by a strong and functionally intact immune response. Moreover, these data suggest that other factors than viral evolution strongly contribute to viral persistence. Recent studies highlighted secondary T cell failure by exhaustion (11, 12) or suppression by regulatory T cells (13, 14).

The results of this study have been published in the Journal of General Virology 2007.

5. Comprehensive analysis of the intrahepatic CD8 T cell response and the impact on viral evolution

To further extend our understanding of the interaction between CD8 selection pressure and viral evolution we also started to determine the intrahepatic HCV-specific T cell response utilizing a comprehensive approach with overlapping peptides in concert with an analysis of the autologous viral sequence. A total of 20 subjects with chronic HCV genotype 1 infection were included (table 1). A liver-biopsy was performed for diagnostic purposes on all subjects, a portion of the obtained material was then used for expansion of intrahepatic T cells as previously described (15). The T cell response was determined by Elispot and compared to the T cell response in PBMC after the same expansion protocol of T cells. All identified epitopes were confirmed by ICS and finemapped where possible. The autologous sequence of all targeted epitopes was determined and if differences from the prototype epitope sequence were present the impact on T cell recognition of the variant autologous sequence was determined.

Table 1: *Targeted CD8 epitopes of 20 subjects with chronic HCV Infection. Frequency (in %) of specific T cells in the intrahepatic compartment (IHL) and in peripheral blood (PBMC) are shown*

Genotype 1a patients:

ID	Epitope	HLA	consensus (autologous)	IHL	PBMC
1	NS2 849-857	A2 #	FLTRVEAQL L--------	0.66	0.06
	NS3 1058-1072	ND #	EVQIVSTAAQTFLAT --------T------	0.16	-
	NS5A 1987-1995	A2	VLSDFKTWL ---------	0.41	-
	NS5B 2594-2602	A2	ALYDVVSKL ---E-----	0.19	-
2	NS3 1053-1061	B38 #	NQVEGEVQI -R--------	0.13	0.23
	NS3 1080-1088	A66 #	TVYHGAGTR --------K	0.23	0.40
	NS3 1191-1208	ND #	KAVDFIPVENLETTMRSP ------------------	0.59	0.16
	NS5A 2184-2192	A66 #	EAAGRRLAR ---R-----	0.98	1.78
3	E2 721-729	A2	LLFLLLADA ---------	-	0.16
	NS5B 2836-2853	ND #	APTLWARMILMTHFFSVL ------------------	-	0.11
4	NS2 957-964	B37	RDWAHNGL -G------	0.59	0.16
	NS5B 2926-2934	A3 #	SVRARLLSR *not done*	0.04	-
5	E1 234-242	B35	NASRCWVAM --------P-	0.02	0.06
	E2 540-549	B57	NTRPPLGNWF -----S----	0.08	-
	E2 589-597	B35 #	HPEATYSRC *not done*	0.29	-
	NS3 1492-1509	ND #	GRGKPGIYRFVAPGERPS ---RR------------	0.10	-
	NS5A 2163-2171	B35	EPEPDVAVL ---------	0.07	-
	NS5B 2629-2637	B57	KSKKTPMGF *not done*	0.07	-
6	E2 661-669	A2 #	ELSPLLLST ---------	-	0.05
7	NS3 1268-1285	ND #	MSKAHGVDPNIRTGVRTI -------E----------	0.18	-
	NS4A 1695-1702	B35	IPDREVLY V-------	0.05	0.48
8	E2 708-716	B58 #	SIASWAIKW -------R-	0.04	-
	NS4B 1744-1754	A24 *	EVITPAVQTNW -----V---S-	0.08	-
9	E2 521-529	B58 #	RSGAPTYSW -A-V---D-	0.69	0.08
	E2 610-618	Cw7	DYPYRLWHY ---------	0.44	0.03
	NS2 974-991	ND #	VVFSRMETKLITWGADTA ------------------	0.26	0.03
	NS3 1596-1604	B58 #	RAQAPPPSW --L------	0.09	-
	NS4B 1745-1754	A24	VIAPAVQTNW A---------	2.37	0.08
	NS4B/5A 1968-1976	B58 #	CTTPCSGSW F-----S--	0.87	0.03
	NS5B 2934-2951	ND #	RGGRAAICGKYLFNWAVR ------T--R--------	0.89	-
10	No epitope identified.				

Genotype 1a patients (continued):

ID	Epitope	HLA	consensus (autologous)	IHL	PBMC
11	Core 35-44	A2	YLLPRRGPRL ----------	4.72	0.31
	E1 358-375	ND #	GIAYFSMVGNWAKVLVVL ------------------	0.11	0.03
	E2 489-496	B51	YPPKPCGI --------	0.10	-
	NS2 849-857	A2 #	FLTRVEAQL L------H-	0.44	0.05
	NS3 1073-1081	A2	CINGVCWTV -------S-	-	0.12
	NS3 1406-1415	A2	KLVALGINAV ----M-V---	0.06	0.03
	NS3 1636-1643	A11	TLTHPITK --------	0.12	-
	NS3 1644-1653	A2	YIMTCMSADL ----------	-	0.58
	NS5B 2575-2582	B7	KPARLIVF --------	0.23	-
12	P7 790-798	A29	LYGMWPLLL A--------	-	0.03
	E1 234-242	B35	NASRCWVAM -T-----P-	-	0.04
	E2 463-480	ND #	TDFDQGWGPISYANGSGP A----------------	0.14	0.07
	NS2 828-836	A3 #	ALTLSPYYK ------H--	0.10	0.11
	NS3 1409-1417	A3 #	ALGINAVAY G--------	0.04	-
13	No epitope identified.				

Genotype 1b patients:

ID	Epitope	HLA	consensus (autologous)	IHL	PBMC
14	E2 541-550	B57 *	NTRPPLGNWF ----------	0.02	-
	NS5A/5B 2416-2424	A26 #	DVVCCSMSY ---------	0.04	-
	NS5B	B27 #	ARMILHTHF V--V-----	0.14	-
15	NS2 910-919	Cw3	PYFVRAQGLI ---------V	0.77	-
	NS5B 2836-2853	ND #	APTLWARMILMTHFFSVL ------------------	0.06	0.13
16	Core 28-37	B60 *	GQIVGGVYLL ----------	0.30	0.05
	Core 41-49	B7	GPRLGVRAT -------T-	0.27	0.10
	NS5B 2568-2577	B55	QPEKGGRKPA -A--------	0.03	-
17	E1 234-242	B35	NASRCWVAM -L-------	0.07	0.09
	E2 610-618	Cw7	DYPYRLWHY ---------	0.30	-
18	Core 43-51	A3	RLGVRATRK ---------	0.05	-
	NS3 1123-1131	A1 *	CTCGSSDLY ---------	0.03	0.34
19	NS3 1031-1039	A24	AYSQQTRGL ---------	-	0.14
20	E2 614-622	A2	RLWHYPCTV ---------	-	0.06
	NS3 1073-1081	A2	CINGVCWTV -----L-S-	0.07	-
	NS3 1268-1285	ND #	MSKAHGVDPNIRTGVRTI ------I--S----I---	0.12	-
	NS5A 1987-1995	A2	VLSDFKTWL ---------	0.06	-

\- tested negativ (<0.02%)
\# newly identified epitope
* epitope previously described, HLA crossrecognition

In 18 of 20 subjects with chronic HCV infection a CD8 T cell response (range 1-9 CD8+ epitopes) was detectable either in the liver or in PBMC. A total of 61 CD8 epitopes was identified, 27 (44%) of these epitopes represent novel specificities that were not previously described. As reported previously HCV-specific T cells were enriched in the liver. Interestingly, only 24 of 61 (39%) epitopes were detected both in the liver as well as in PBMC (Fig. 1). 46% of all epitopes were detected only in the liver but not in PBMC indicating that these responses were absent or below the threshold of detection in the peripheral blood. 15% of responses were only detectable in PBMC.

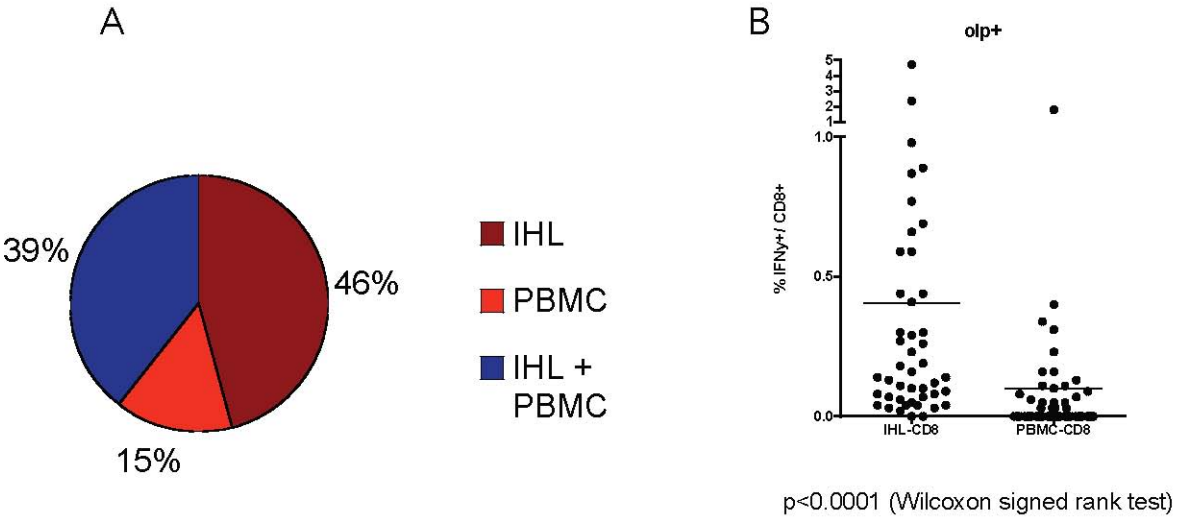

Fig. 1: A. *Distribution of HCV-specific CD8+ T cell responses in liver (IHL) and blood (PBMC).* ***B****. Strength of HCV-specific CD8+ T cell responses in liver and blood.*

The autologous sequence of the targeted CD8 epitope was obtained for 58 of 61 epitopes. A genotype 1a and a genotype 1b consensus sequence was calculated as the most predominant amino acid for each position in the polyprotein based on 80 genotype 1b full genomes available at the LANL database (www.hcv.lanl.gov) and 70 genotype 1a full genomes from a Bostoner cohort (16). We hypothesize that differences from the consensus sequence are potentially driven by immune selection pressure. In 34 of 58 cases the autologous sequence differed from the corresponding consensus sequence suggesting the possibility of mutational escape in these regions. To determine the impact of these observed variants on T cell recognition, peptides corresponding to the autologous sequence were synthesized and tested in comparison to the prototype sequence in serial dilutions by intracellular cytokine staining (ICS) for interferon gamma. A total of 21 variant peptides were tested. T cell recognition of the autologous sequence was substantially decreased in 17 of 21 cases consistent with escape (example in figure 2). Though not formally proven, these data suggest that escape mutations are selected in 50% of CD8 epitopes targeted by intrahepatic T cells. This finding is in line with previous observations during acute HCV infection in which the T cell responses were determined in PBMC (8).

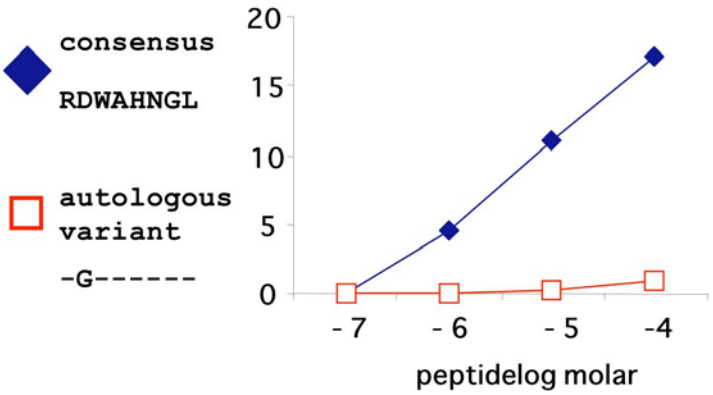

Fig. 2: *Functional titration of consensus peptide (blue diamond) versus autologous variant peptide (red box). As an example, epitope NS2 957-964*.

The results of this study were presented at the 13th International Symposium on Hepatitis C

Virus and related viruses in an oral presentation and a manuscript for publication is in review.

Analysis of the evolutionary forces in an immunodominant CD8 epitope in the hepatitis C virus at a population level

For the previous study we hypothesized that differences from the consensus sequence as the most common residue for each position in the HCV protein are potentially driven by immune selection pressure. This is an accepted approach to determine positive (away from consensus) and negative (towards consensus) selection in viral evolution (17). However, if the virus rarely reverts back to the consensus residue upon transmission in the absence of immune pressure, escape mutations can accumulate and become the predominant residue in a population. The kinetics of mutational escape therefore not only depends on the frequency and strength of the immune response. Possible fitness costs associated with escape mutations are also a contributing factor to viral evolution. To address this interaction we studied a frequently targeted HLA-A1-restricted epitope in a cohort infected with genotype 1b and 3a. Mutational escape from the prototype (ATDALMTGY) to a variant (ATDALMTGF) has been described during acute infection. Sequence analysis of subjects with chronic HCV infection revealed that the predominant sequence in this region is the putative escape variant (figure 4). Of note, the Y1444F substitution is associated with the presence of the HLA-A1 allele. However, even in the absence of the HLA-A1 allele the Y1444F substitution is highly prevalent suggesting that this substitution is accumulating in the population.

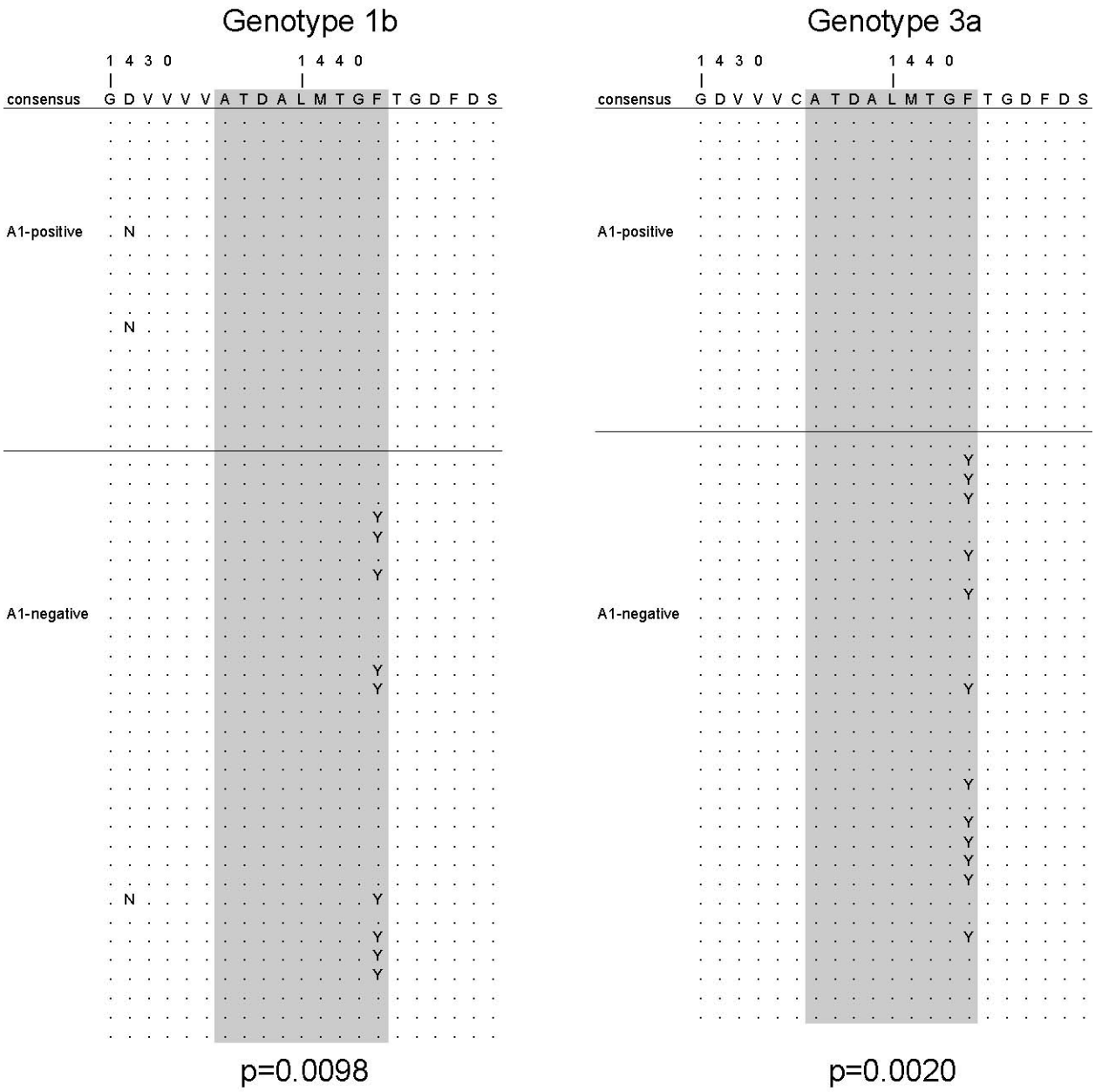

Fig. 4: *Sequences from subjects with chronic HCV genotype 1b and genotype 3a infection. The prototype sequence is shown on top. Matches with the prototype sequence are indicated with a dot. In the upper part sequences from HLA-A1 positive subjects are shown, in the lower part sequences from HLA-A1 negative*

To determine the frequency of this HLA-A1 restricted epitope in subjects with chronic infection 10 subjects infected with HCV genotype 1b and 10 subjects infected with genotype 3a were analyzed. After in-vitro expansion of antigen-specific T cells the response was detectable in 5/10 subjects infected with genotype 1b and 4/10 subjects infected with genotype 3a (data not shown). This frequency during the chronic phase of infection is similar to the observed frequency of other immunodominant epitopes.

Fig. 5: Accelerated off-rate of the Y1444F variant. *HLA-A*01 positive allogeneic Epstein–Barr virus (EBV) immortalized B cell lines (B-LCL) were incubated without peptide (left panel) or in the presence of the prototype peptide (middle panel) or the Y1444F variant (right panel). After washing six times with medium the B cells were used as targets for polyclonally expanded T cells in an ICS for IFN-γ. Dot plots from two subjects (1b-Ch5 and 3a-Ch7) are shown.*

We next determined the impact of the observed sequence variation on T cell recognition. In all 9 subjects where this immune response was detected we were able to reproducibly expand T cells specific for this HLA-A1 epitope after incubation with the prototype antigen. Interestingly, in most cases the polyclonally expanded T cell line showed substantial crossreactivity with the putative escape variant. However, after incubation with the variant (Y1444F) no specific T cells were detectable in any of these subjects, suggesting that the variant sequence did not stimulate proliferation of antigen-specific T cells. The mechanism of this difference was probably an accelerated off-rate of the variant peptide. When B cells were pulsed with peptide and washed extensively, they were only targeted when they were pulsed with the prototype. The variant peptide was not presented anymore (Figure 5) supporting that the Y1444F substitution truly acts as an escape mutation.

In collaboration with David Frick from the Department of Biochemistry & Molecular Biology at the New York Medical College the functional impact of this polymorphisms on the encoded protein was determined. The mutation is located in the helicase domain of NS3. As expected based on the observation that the Y1444F substitution is quite frequent also in the absence of immune pressure the impact of this polymorphism on helicase activity is relatively small. However, the activity of different helicases harbouring the prototype residue in position 1444 (tyrosine) was reproducibly slightly higher. This probably prevents continuous accumulation and ultimately deletion of this epitope from the population.

Interestingly, the frequency of the Y1444F substitution in circulating HCV genotype 1b isolates varies between different regions of the world. In Europe, Australia and the US the escape variant is predominant (table 2). However, in Asia the most frequent observed isolate is the prototype.

Table 2: *Frequency of the Y1444F polymorphism in HCV genotype 1b in different populations*

Germany		Europe	Asia	USA	Australia	Rest
n sequences	49	35	35	17	12	1
Y/F	9/40	4/31	20/15	5/12	5/7	0/1
% F	81.6	88.6	42.9	70.6	58.3	-
consensus	F	F	Y	F	F	-

This correlates with the HLA-A1 frequencies in these populations. HLA-A1 is one of the most frequent alleles in Europe but is rare in Asian populations (figure 6). This suggests that the frequency of the Y1444F escape mutation in the immunodominant CD8+ epitope in HCV NS3 is substantially influenced by the frequency of the restricting allele in a population. This has important consequences for the ability to prime a functional immune response against a virus that is already adapated in this important antigen and may be relevant for future vaccine designs.

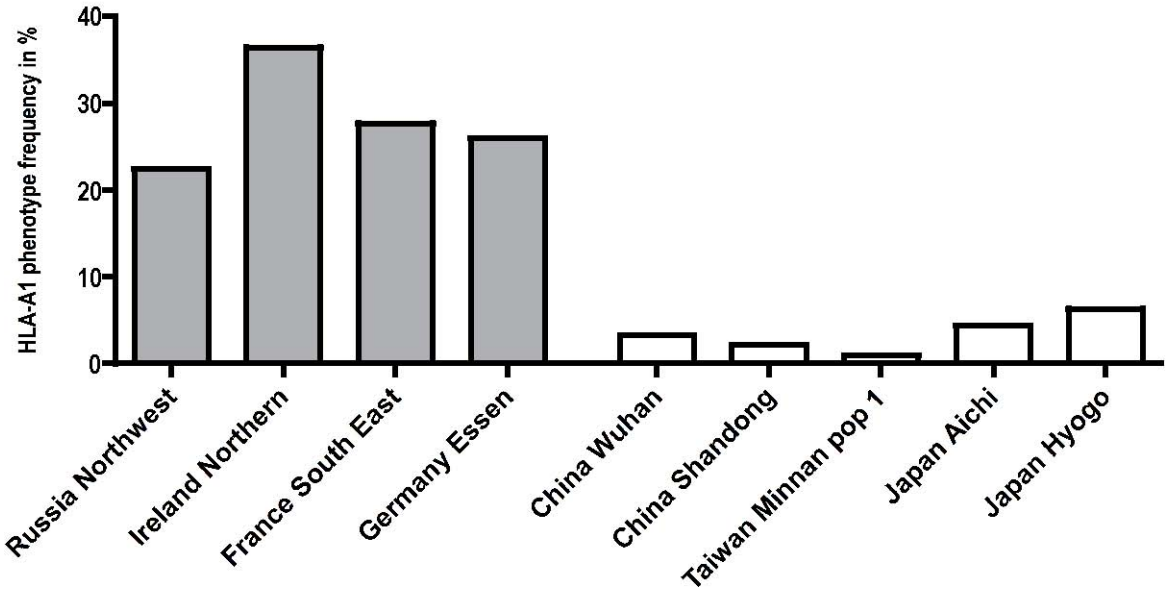

Fig 6.: *Frequency of the HLA-A1 allele in different cohorts.*
The results of this project were presented at the Annual Meeting of the American Association for the Sudy of Liver Disease 2006 and a manuscript for publication is in review.

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