Abschlussbericht Teilprojekt 15.4

Projekttitel: Mechanismen der Hepatitis C-Virus Persistenz: Viraler Escape versus T-Zell Dysfunktion

Projektleiter1: PD Dr. med. R. Thimme
Universitätsklinik Freiburg
Innere Abteilung II
Hugstetter Str. 55
79106 Freiburg

Projektleiter2: PD Dr. S. Viazov
Universitätsklinikum Essen
Institut für Virologie
Hufelandstr. 55
45122 Essen

Telefon: +49(0) 761-270 3758

Fax: +49(0) 761-270 3372

E-Mail: thimme@med1.ukl.uni-freiburg.de

Berichtszeitraum: 01.02.2002 – 31.12.2004

Summary

T- lymphocytes are thought to play a major role in the elimination of hepatitis C virus (HCV) infection. Acutely infected patients, that clear the virus, usually mount a strong and multispecific T cell response whereas in patients with chronic HCV infection only weak and narrowly focused T cell responses are detectable in the blood [18]. The immunological basis for the failure of the T cell response to control or eliminate the virus during chronic infection is not known. Recent studies have indicated that virus-specific CD8+ T cells are impaired in their effector functions, e.g. cytokine secretion, cytotoxicity and proliferation [5, 17, 19]. It has also been suggested that the early development of viral escape mutations may be an important determinant of HCV persistence [3, 20]. Importantly, most of these studies have been performed with peripheral blood lymphocytes and not with liver infiltrating lymphocytes. Since the virus replicates primarily in the liver and since it is not known if the frequency, phenotype and function of virus-specific CD8+ T cell response in the blood is the same as in the liver, this study was designed to analyze the intrahepatic T cell response and correlate it with the corresponding viral sequence present in the patient. These results should give further information about the relative contribution of dysfunction and/ or viral escape in viral persistence during chronic HCV infection.

2. Present standard of knowledge

There is a growing consensus that the development of a relatively strong peripheral CD4+ and CD8+ T cell response to HCV infection correlates with the resolution of infection [13]. The CD8+ T cells are thought to be the main effector cells which has been most clearly demonstrated in experimentally infected chimpanzees [1, 3, 15]. In these studies it has been shown that a multispecific intrahepatic HCV-specific CD8+ T cell response during the early phase of infection was associated with viral clearance while a more narrowly focused and delayed response was associated with persistent infection and the emergence of viral escape mutations. In humans several groups have observed a strong CD8+ T cell response in clinically symptomatic acutely infected patients who clear the infection; however, the durability of that response after recovery is variable in different studies [2, 6, 9-11, 14, 16]. In chronically infected patients, there is a general agreement that HCV specific CD8+ T cells are present in the blood. Controversial results about the detection of

intrahepatic virus-specific CD8+ T cells have been reported. While several groups have described the detection of intrahepatic T cells [4, 7], a recent study was unable to detect HCV-specific CD8+ T cells in a large cohort of patients [12]. It is widely assumed that CD8+ T cells during chronic infection are sufficient to maintain ongoing liver inflammation but unable to eliminate the virus. Importantly, no information is currently available about the function of HCV tetramer positive CD8+ T cells in the liver. In addition, the virus-specific CD8+ T cell response has not been correlated with the actual viral sequence present in the patient, in order to determine possible CTL escape mutations and prove the concept of dysfunction (which is only possible when it is demonstrated that peptides used in the study mirror the viral epitope sequence). Therefore the aim of this study is combine the analysis of effector functions of virus-specific CD8+ T cells with the analysis of the viral sequence to better understand the viral-host interactions that determine viral persistence during chronic infection.

3. Overall aim of this study

It was the overall aim of this study to characterize the host-viral interactions at the site of infection and hereby to better understand the immunological and virological mechanisms that are responsible for HCV persistence during chronic infection. A detailed analysis of the virus-specific CD8+ T cells in the liver and blood and an analysis of the viral sequences in the region of various CTL epitopes was planned to be performed in at least 10 HLA A2 positive chronic HCV infected patients to correlate the T cell phenotype and function with the in vivo HCV sequence. The same analysis except of the intrahepatic T cells was planned to be performed in at least 10 chronic HLA A2 positive chronic HCV patients 18-20 years after documented exposure to an accidently HCV (genotype 1b)-contaminated human Rhesus immunoglobulin [21]. Since all of the patients have been infected with the same inoculum, this will allow to determine the frequency and possible role of viral escape mutations in previously described immunodominant epitopes. The overall goal of this study is to allow a better understanding of the following questions:

Are virus-specific CD8+ T cells detectable in the liver of the majority of chronically HCV infected patients?

Are the intrahepatic virusspecific CD8+ T cells functional or dysfunctional (cytokine secretion, cytotoxicity, proliferation)?

Differs the functional phenotype of intrahepatic HCV specific CD8+ T cells from the

functional phenotype in the blood?
Are the viral sequences corresponding to CTL epitopes used in this study variant and could therefore represent viral escape mutations?
What is the relative contribution of dysfunction and viral escape during chronic HCV infection?

In sum, the results of this study should provide important insights into the immunobiology and pathogenesis of HCV infection and should help to understand the viral and immunological reasons for the inefficent T cell response during chronic HCV infection. Furthermore, these results may suggest new therapeutic approaches designed to focus the antiviral power of the T cell response (e.g. virus-specific immunotherapy) at the site of viral replication in persistently infected patients, thereby reducing the risk of chronic liver disease and hepatocellular carcinoma.

4. Initially proposed work program and methods

We planed to study the peripheral and intrahepatic CD8+ T cell response in 10 HLA-A2 positive patients with chronic HCV infection as well as the peripheral CD8+ T cell response in 10 HLA-A2 positive patients from a well documented HCV exposure 18-20 years ago.
Blood and liver biopsies from the same timepoint were be obtained. Liver infiltrating lymphocytes (LIL) were isolated from approximately 0.5-1 cm of hepatic needle biopsy. The tissue was homogenized in 2-3 ml of PBS using a Dounce tissue grinder. Cell suspensions were then incubated with beads coupled to anti-CD8 or anti-CD4 antibodies (Dynabeads) for 20 minutes and bound CD8+ or CD4+ T cells were isolated using a particle magnetic concentrator. These isolated intrahepatic CD4+ and CD8+ T cells were then plated into separate wells in 24 well plates in 1 ml of 10% FCS supplemented with IL2 and anti-human CD3 monoclonal antibody as a stimulus for T cell growth and 2 x 106 irradiated autologous PBMC as feeders. After 2-3 weeks, the expanded T cells were tested for HCV specific responses.
HCV specific CD8+ T cell responses were tested by HLA-A2 tetramers corresponding to 4 different HCV peptides (Table 1) that have been previously described. Indeed, as recently described by Lauer at al. [8] three of these four peptides have been shown to be immunodominant. For these assays, T cells were incubated for 30 min with 1 µg of the APC labeled tetramers in a 96 well v-bottom

plate. Cells were washed in phosphate-buffered saline containing 1% FCS and incubated with anti CD8 and other surface markers for 30 minutes. Cells were washed again 3 times by consecutive resuspension in 250 µl (total volume/well) of staining buffer, and then resupended in 200 µl PBS with 2% paraformaldehyde before FACS analysis.

Intracellular IFN-□ staining was performed as described. Briefly, T cells (0,5-2x106 cells/ml) were stimulated with peptide antigens (10 µg/ml) in duplicate wells in the presence of 50U/ml human rIL-2 (Hoffmann-La Roche, Inc., Nutley, N.J.) and 1 µl/ml Brefeldin A (Pharmingen, San Diego, CA). After 5 hours of incubation (37o C, 5% CO2), the cells from each well were stained with antibodies to CD8 and IFN□ (Pharmingen, San Diego, CA). Duplicate wells without peptide were also included to determine the background level of IFN□ production. In addition, one well without peptide was included for isotype antibody staining, and another well was stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co, St Louis, MO) and 200 ng/ml ionomycin to serve as a positive control for IFN□ staining. The frequency of cytokine-positive CD8+ T cells was defined as the difference between the frequency detected in peptide-stimulated and unstimulated cells.

Sequence analysis of CTL epitopes was performed as follows: Following serum RNA extraction, RT-PCR, and cDNA cloning, the predominant clonotype was determined by a single-stranded conformational polymorphism analysis of the genome regions corresponding to epitopes of interest. For such an analysis of the quasispecies population the variant of the temperature gradient gel electrophoresis (TGGE) developed in our laboratory was used. In case of the evident polymorphism the identified clones were subjected to bi-directional sequencing. In cases of putative escape-mutations within the tested epitopes, corresponding variant peptides were synthesized. T cells were then stimulated with wild type as well as with “mutant” peptides and stained for peptide specific intracellular IFN□ production as described above.

Achievements and results

We were able to perform the analysis of virus-specific T-cells in the peripheral blood and in the liver of HLA-A2 positive chronic HCV infected patients as proposed in the work program. 12 HLA-A2 positive chronically HCV infected patients (HCV antibody

and HCV-PCR positive) were enlisted into this study. Blood samples were drawn for serological, virological, and immunological analysis and the medical history of each subject was recorded. A summary of the patients characteristics is provided in 2. The study protocol was approved by the local ethics committee. The results of the study can be summarized as follows.

Frequency of intrahepatic HCV specific CD8+ T cells in chronic HCV infection

We determined the frequency of HCV specific CD8+ T cells in expanded intrahepatic CD8+ T cells from 12 HLA-A2 positive patients with chronic HCV infection. The expanded cells were screened for the presence and frequency of HCV specific CD8+ T cells by using four well defined HLA-A2 tetramers (Table 1). HCV specific CD8+ T cell responses were detectable in 10 of the 12 patients (83%). These responses targeted an average of 2,5 epitopes (0-4), primarily within the non-structural region.

Overlap between the peripheral and intrahepatic CD8+ T cell response

Peripheral blood mononuclear cells obtained at the same time as the intrahepatic lymphcytes were also studied to compare the frequency of HCV-specific CD8+ T cells in the peripheral blood and the intrahepatic compartment. Peripheral HCV specific CD8+ T cell responses were detectable in 9 of the 12 patients (75%), targeting an average of 1,5 epitopes (range 0-3). All HCV-specific T cells targeted the non-structural region, however, the T cell epitope repertoire overlapped significantly between both compartments. The frequency of HCV specific CD8+ T cells was quite low and was significantly lower compared to the intrahepatic compartment. In contrast, Flu specific CD8+ T cells were detectable in the peripheral blood of most patients and had a higher median frequency as compared with the intrahepatic compartment, suggesting that HCV but not Flu specific CD8+ T cells are specifically recruited to the HCV infected liver.

Function of intrahepatic CD8+ T cells in chronic HCV infection

Next, we determined the ability of intrahepatic HCV specific CD8+ T cells to produce IFN after stimulation with the corresponding peptide. To investigate the IFN production of the intrahepatic CD8+ T cells we performed intracellular cytokine staining assays with the expanded intrahepatic CD8+ T cells. As shown in Figure 1B, only a minority of intrahepatic tetramer positive HCV specific CD8+ T cells secreted

IFNy after specific stimulation with the cognate peptide suggesting that a large fraction of CD8+ T cells is impaired in their ability to secrete IFN at the site of disease, the liver. Notable, this dysfunction was observed despite the IL-2 supplementation of these cells in vitro. Representative results from two of the patients are shown in Figure 1A. In contrast to the dysfunction of most intrahepatic HCV specific CD8+ T cells, Flu specific CD8+ T cells produced IFN in all but one patient (Fig. 1B). These results clearly indicate that dysfunction is not a general phenomena of intrahepatic virus-specific CD8+ T cells but rather specific for HCV cells. Finally, it is also important to note, that the impaired ability of HCV-specific CD8+ T cells to produce IFN was not specific to the intrahepatic compartment, because the same phenomena was observed with HCV-specific CD8+ T cells present in the peripheral blood.

Intrahepatic CD8+ T cell failure: T cell dysfunction and viral escape

Next, we correlated the viral peptide sequences present in a given patient with the corresponding prototype peptide specific intrahepatic CD8+ T cell response. Interestingly, as shown in Table 4, 10 virus-specific CD8+ T cell responses were observed that did not produce IFN. In five of the corresponding viral epitopes we observed sequence variations (50%) indicating that dysfunction in at least half of the patients was not due to a mismatch of peptides used to study IFN secretion of these T cells. Indeed, especially in the regions of the Core132 and NS31073 epitope, functional HCV-specific CD8+ T cell responses were observed in several cases where the epitope did not undergo mutation in the chronic phase of infection.

It is also important to note that a total of 9 intrahepatic CD8+ T cell responses were detectable in the same patient group that secreted IFN Notably, six of the corresponding autologous viral sequences displayed variations in the corresponding region (66,6%). In order to test the hypothesis that some of these variant peptides are potentially CTL escape variants, the variant NS3 1406 peptides from patients 1, 7 and 8 were synthesized and tested for CD8+ T cell cross-reactivity and immunogenicity. Importantly, as shown in Figure 2, no recognition of the variant was observed in patients 1 and 7 across a range of peptide concentrations (0,1 µg – 10µg), compatible with CTL escape at these loci. However, it is also important to note that the T cell response against the genotype 1a peptide sequence in patient 1 may represent a residue from a previous resolved infection with a genotype 1a strain. In

the case of patient 8, however, recognition of the variant was observed, also weaker compared to the prototype, excluding viral escape.

The two variant peptide sequences most often observed in the NS52594 epitope, that probably do not reflect viral escape but the infecting genotype, were both recognized, excluding viral escape and suggesting that the dysfunction observed in patient 4 is not due to a mismatch of peptides used in the study. Unfortunately, no cells were left from patient 7 to perform additional studies. It is also important to note that in the case of the NS31073 epitope, functional HCV-specific CD8+ T cell responses were observed in several cases where the epitope did not undergo mutation in the chronic phase of infection (patients 5, 10). These results suggest that intrahepatic CD8+ T cell dysfunction and viral escape mutations both contribute to the failure of the intrahepatic CD8+ T cell response to control HCV replication and that several epitopes do not undergo mutation despite the presence of a functional T cell response. Thus, viral escape is not a universal mechanism of viral persistence during chronic HCV infection.

CD8+ T cell response in 10 HLA-A2 positive patients from a well documented HCV exposure

Due to logistical problems and difficulties in patient recruitment we were not able to analyze the peripheral CD8+ T cell response in 10 HLA-A2 positive patients from a well documented HCV exposure 18-20 years ago, so far. However, we will get the samples soon and will report of the findings separately from this final report.

Publications

Part of the results of this project have been present at the international HCV meeting in Kyoto, Japan (oral presentation) and at the AASLD meeting in Boston 2003. A manuscript with the title ”Intrahepatic CD8+ T cell failure during chronic HCV infection” will be submitted soon.

Summary

CD8+ T cells are thought to control hepatitis C virus infection in the acute phase of infection. Virus-specific CD8+ T cells are also detectable in the chronic phase of infection. The precise mechanims responsible for failure of intrahepatic HCV-specific CD8+ T cells to control the virus during persistent inection have not been fully

defined. To address this important issue we have studied the intrahepatic and peripheral CD8+ T cell response in 12 HLA-A2 chronically HCV-infected patients by using four previously well defined HLA-A2 restricted epitopes. The intrahepatic HCV-specific CD8+ T cell response was compared with the corresponding sequences encoded by the the infecting viruses. The results of the study can be summarized as follows. First, intrahepatic HCV-specific CD8+ T cells are present in the liver of a majority of chronically HCV infected patients. This response overlaps significantly with the response present in the peripheral blood although it is enriched in the intrahepatic compartment. Seond, a large fraction of intrahepatic HCV-specific CD8+ T cells are impaired in their ability to secrete IFN□. This dysfunction is specific for HCV-specific CD8+T cells since Flu-specific CD8+ T cells readily secrete this cytokine. Third, viral escape may have occurred in some of the patients before their entrance into the study. However, in several instance no variation was observed despite the presence of prototype or cross-immunogenic intrahepatic CD8+ T cell responses suggesting that viral escape is not an universal but rather rare and epitope specific cause of intrahepatic T cell failure. Taken together, our results suggest that different mechanisms contribute to the failure of intrahepatic CD8+ T cells to sufficiently control the virus, despite their survival and accumulation in the infeced organ.

Literature

1Cooper, S, Erickson, AL, Adams, EJ, Kansopon, J, Weiner, AJ, Chien, DY, Houghton, M, Parham, P,Walker, CM. Analysis of a successful immune response against hepatitis C virus. Immunity 1999; 10: 439-449.

2Diepolder, HM, Zachoval, R, Hoffmann, RM, Wierenga, EA, Santantonio, T, Jung, MC, Eichenlaub, D,Pape, GR. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 1995; 346: 1006-1007.

3Erickson, AL, Kimura, Y, Igarashi, S, Eichelberger, J, Houghton, M, Sidney, J, McKinney, D, Sette, A, Hughes, AL,Walker, C. The outcome of hepatitis C virus infection is predicted by escape mutaions in epitopes targeted by cytotoxic T lymphocytes. Immunity 2001; 15: 885-895

4Grabowska, AM, Lechner, F, Klenerman, P, Tighe, PJ, Ryder, S, Ball, JK, Thomson, BJ, Irving, WL,Robins, RA. Direct ex vivo comparison of the breadth and specificity of

the T cells in the liver and peripheral blood of patients with chronic HCV infection. Eur J Immunol 2001; 31: 2388-2394.

5Gruener, NH, Lechner, F, Jung, MC, Diepolder, H, Gerlach, T, Lauer, G, Walker, B, Sullivan, J, Phillips, R, Pape, GR,Klenerman, P. Sustained dysfunction of antiviral cd8(+) t lymphocytes after infection with hepatitis c virus. J Virol 2001; 75: 5550-5558.

6Gruner, NH, Gerlach, TJ, Jung, MC, Diepolder, HM, Schirren, CA, Schraut, WW, Hoffmann, R, Zachoval, R, Santantonio, T, Cucchiarini, M, Cerny, A,Pape, GR. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. J Infect Dis 2000; 181: 1528-1536.

7He, XS, Rehermann, B, Lopez-Labrador, FX, Boisvert, J, Cheung, R, Mumm, J, Wedemeyer, H, Berenguer, M, Wright, TL, Davis, MM,Greenberg, HB. Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. Proc Natl Acad Sci U S A 1999; 96: 5692-5697.

8Lauer, GM, Nguyen, TN, Day, CL, Robbins, GK, Flynn, T, McGowan, K, Rosenberg, ES, Lucas, M, Klenerman, P, Chung, RT,Walker, BD. Human immunodeficiency virus type 1-hepatitis C virus coinfection: intraindividual comparison of cellular immune responses against two persistent viruses. J Virol 2002 Mar; 76: 2817-2826.

9Lechner, F, Gruener, NH, Urbani, S, Uggeri, J, Santantonio, T, Kammer, AR, Cerny, A, Phillips, R, Ferrari, C, Pape, GR,Klenerman, P. CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. Eur J Immunol 2000; 30: 2479-2487.

10Lechner, F, Wong, DK, Dunbar, PR, Chapman, R, Chung, RT, Dohrenwend, P, Robbins, G, Phillips, R, Klenerman, P,Walker, BD. Analysis of successful immune responses in persons infected with hepatitis C virus. J Exp Med 2000; 191: 1499-1512.

11Missale, G, Bertoni, R, Lamonaca, V, Valli, A, Massari, M, Mori, C, Rumi, MG, Houghton, M, Fiaccadori, F,Ferrari, C. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J Clin Invest 1996; 98: 706-714.

12Prezzi, C, Casciaro, MA, Francavilla, V, Schiaffella, E, Finocchi, L, Chircu, LV, Bruno, G, Sette, A, Abrignani, S,Barnaba, V. Virus-specific CD8(+) T cells with type 1 or type 2 cytokine profile are related to different disease activity in chronic hepatitis C virus infection. Eur J Immunol 2001 Mar; 31: 894-906.

13Rehermann, B,Chisari, FV. Cell mediated immune response to the hepatitis C virus. Curr Top Microbiol Immunol 2000; 242: 299-325

14Takaki, A, Wiese, M, Maertens, G, Depla, E, Seifert, U, Liebetrau, A, Miller, JL, Manns, MP,Rehermann, B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med 2000; 6: 578-582.

15Thimme, R, Bukh, J, Spangenberg, HC, Wieland, S, Pemberton, J, Steiger, C, Govindarajan, S, Purcell, RH,Chisari, FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proc Natl Acad Sci U S A 2002 Nov 26; 99: 15661-15668.

16Thimme, R, Oldach, D, Chang, KM, Steiger, C, Ray, SC,Chisari, FV. Determinants of Viral Clearance and Persistence during Acute Hepatitis C Virus Infection. J Exp Med 2001; 194: 1395-1406.

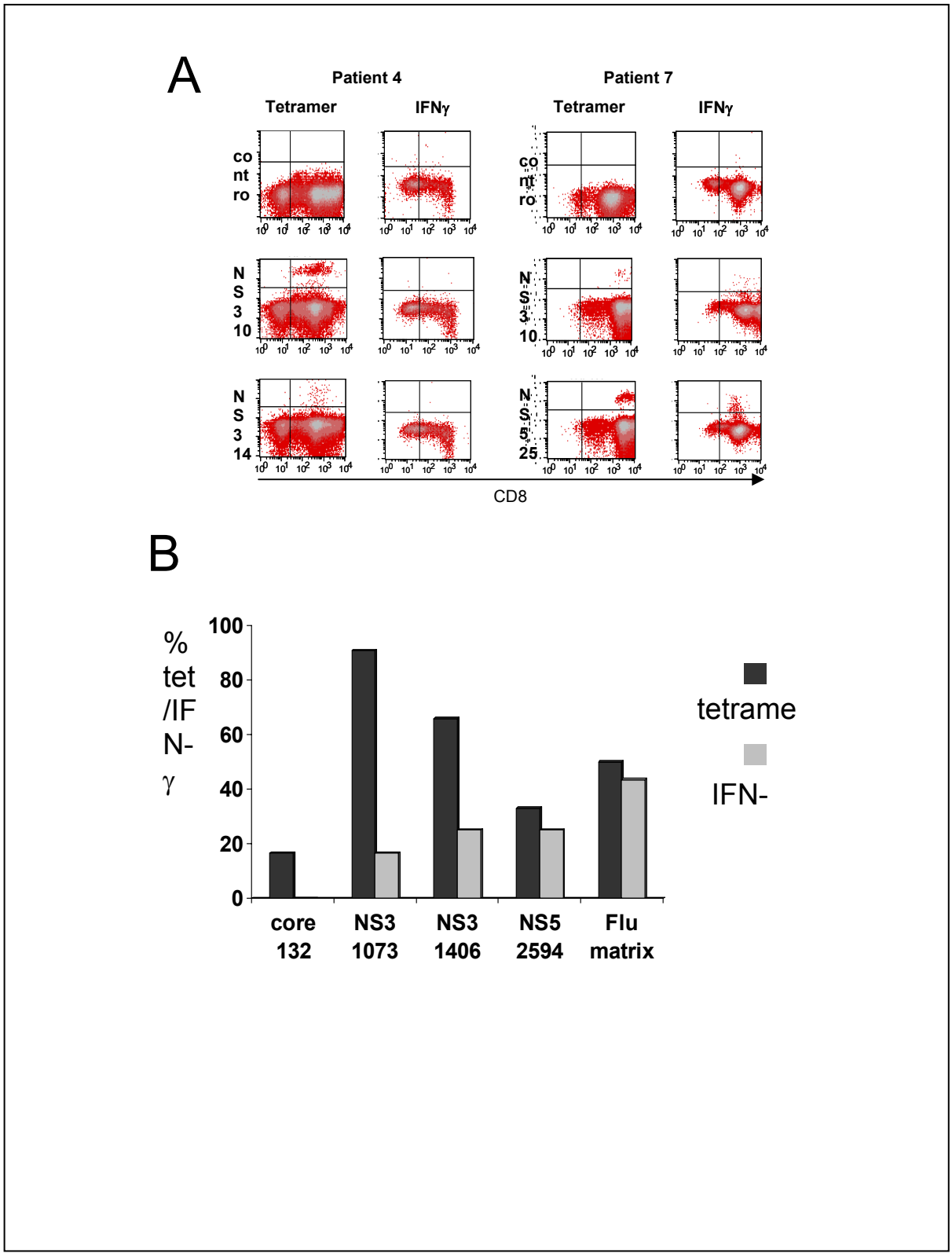
17Urbani, S, Boni, C, Missale, G, Elia, G, Cavallo, C, Massari, M, Raimondo, G,Ferrari, C. Virus-specific CD8+ lymphocytes share the same effector-memory phenotype but exhibit functional differences in acute hepatitis B and C. J Virol 2002 Dec; 76: 12423-12434.

18Ward, S, Lauer, G, Isba, R, Walker, B,Klenerman, P. Cellular immune responses against hepatitis C virus: the evidence base 2002. Clin Exp Immunol 2002 May; 128: 195-203.

19Wedemeyer, H, He, XS, Nascimbeni, M, Davis, AR, Greenberg, HB, Hoofnagle, JH, Liang, TJ, Alter, H,Rehermann, B. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J Immunol 2002 Sep 15; 169: 3447-3458.

20Weiner, A, Erickson, AL, Kansopon, J, Crawford, K, Muchmore, E, Hughes, AL, Houghton, M,Walker, CM. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proc Natl Acad Sci U S A 1995; 92: 2755-2759.

21Wiese, M (1991):Virushepatitis: Forschung und Praxis. In:Non-A-non-B-Hepatitis . Stuttgart: Gustav Fischer Verlag.


A
Patient 4
Patient 7
Tetramer
IFNγ
Tetramer
IFNγ
control
NS3 10
NS3 14
NS5 25
CD8
B
% tet/IFN-γ
100
80
60
40
20
0
core 132
NS3 1073
NS3 1406
NS5 2594
Flu matrix
tetrame
IFN-

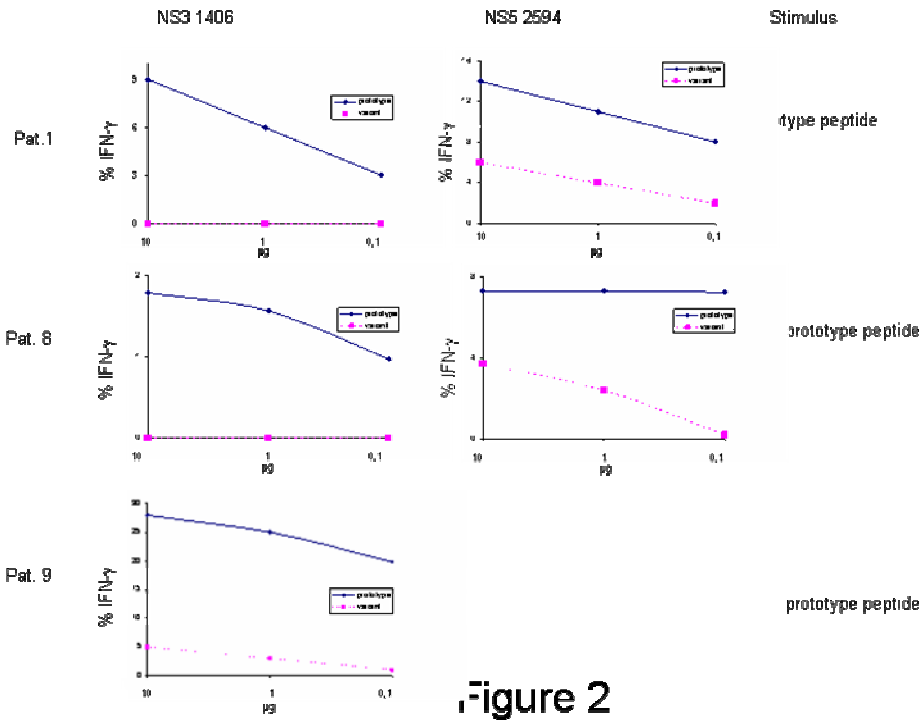

NS3 1406
NS5 2594
Stimulus
Pat.1
Pat. 8
Pat. 9
% IFN-γ
prototype
variant
type peptide
prototype peptide
prototype peptide

Figure 2

Table 1. HLA-A2 Restricted Epitopes

Virus	AA sequence	AA position
HCV	DLMGYIPLV	core 132
	CINGVCWTV	NS3 1073
	KLVALGINAV	NS3 1406
	ALYDVVTKL	NS5 2594
Influenza	GILGFVFTL	Matrix 58

Table 2 Study population

Pts	Age	Sex	ALT	Genotype	viral load	Histology
1	48	F	50	1	1,90E+06	II
2	43	F	25	2	7,00E+06	I
3	40	F	26	1	5,30E+06	II
4	42	M	62	1	7,90E+06	III
5	64	F	162	1	9,50E+06	II
6	41	M	45	1	6,60E+05	II
7	41	M	239	1	2,90E+07	I
8	37	F	78	1	5,10E+05	0
9	34	M	39	1	2,60E+06	I
10	38	M	54	1	2,50E+07	I
11	47	F	111	1	1,60E+06	II
12	23	M	12	1	4,90E+06	II

Table 3

	PBMCs %					IHL %				
Pts	**$Core_{132-140}$**	**$NS3_{1073-1081}$**	**$NS3_{1406-1415}$**	**$NS5_{2594-2602}$**	**Flu**	**$Core_{132-140}$**	**$NS3_{1073-1081}$**	**$NS3_{1406-1415}$**	**$NS5_{2594-2602}$**	**Flu**
1	< 0,03	< 0,03	**0,19**	**0,03**	**0,17**	< 0,03	**0,14**	**0,06**	**0,08**	< 0,03
2	< 0,03	**0,27**	**0,03**	**0,11**	**0,05**	< 0,03	**0,37**	**0,19**	**0,07**	< 0,03
3	< 0,03	< 0,03	< 0,03	< 0,03	n.d.	< 0,03	< 0,03	< 0,03	< 0,03	**0,27**
4	< 0,03	**0,08**	< 0,03*	< 0,03	< 0,03	< 0,03	**1,20**	**0,26**	< 0,03	< 0,03
5	< 0,03	**0,13**	< 0,03	< 0,03	**0,38**	< 0,03	**0,06**	< 0,03	< 0,03	**0,03**
6	< 0,03	**0,13**	< 0,03	< 0,03	n.d.	< 0,03	**0,16**	**0,21**	< 0,03	**0,17**
7	< 0,03	**0,12**	< 0,03	**0,04**	**0,09**	< 0,03	**0,06**	< 0,03	**0,56**	**0,11**
8	< 0,03	< 0,03*	< 0,03*	**0,21**	**0,05**	< 0,03	**0,04**	**0,06**	**0,03**	n.d.
9	< 0,03	< 0,03*	**0,09**	< 0,03	n.d.	**0,09**	**0,4**	**0,76**	< 0,03	n.d.
10	< 0,03	< 0,03	< 0,03*	< 0,03	**0,08**	< 0,03	**0,03**	**0,04**	< 0,03	**0,06**
11	< 0,03	**0,28**	**0,05**	< 0,03	**0,07**	< 0,03	**1,23**	**0,03**	< 0,03	< 0,03
12	< 0,03	< 0,03	< 0,03	< 0,03	< 0,03	**0,04**	**0,1**	< 0,03	< 0,03	< 0,03
□ of	0	6	4	4	7	2	11	8	4	5
mean	**0**	**0,146**	**0,09**	**0,097**	**0, 127**	**0,065**	**0,344**	**0,2**	**0,185**	**0,128**
% of	**0**	**50**	**33**	**33**	**70**	**16,6**	**91**	**66**	**33**	**50**

Table 4

wt	DLMGYIPLV	CINGVCWTV	KLVALGINAV	ALYDVVTKL	genotype
Pat. 1	---------	-V------	--SG-L----	------ST-	1b
% tet	< 0,03	**0,24**	**0,06**	**0,08**	
%CD8/IFNy	< 0,03	< 0,03	**0,03**	**0,05**	
Pat. 4	---------	---------	----M-V---	------S--	1a
% tet	**0,4**	**2,7**	**0,26**	**1,7**	
% CD8/IFNy	< 0,03	< 0,03	< 0,03	< 0,03	
Pat. 5	---------	---------	--SA--L---	------ST-	1b
% tet	< 0,03	**0,06**	< 0,03	< 0,03	
% CD8/IFNy	< 0,03	**0,03**	< 0,03	< 0,03	
Pat. 6	---------	-V-------	--SG-----I	------ST-	1b
% tet	< 0,03	**0,16**	**0,21**	< 0,03	
% CD8/IFNy	< 0,03	< 0,03	< 0,03	< 0,03	
Pat. 7	---------	---------	------V---	---E--S--	1a
% tet	< 0,03	**0,06**	< 0,03	**0,56**	
% CD8/IFNy	< 0,03	**0,03**	< 0,03	**0,22**	
Pat. 8	---------	---------	---G--L---	------S--	1a
% tet	< 0,03	**0,04**	**0,06**	**0,03**	
% CD8/IFNy	< 0,03	**0,04**	**0,03**	**0,03**	
Pat. 9	---------	---------	---V------	------S--	1a
% tet	0,09	**0,4**	**0,76**	< 0,03	
% CD8/IFNy	< 0,03	< 0,03	**1,3**	< 0,03	
Pat. 12	---------	----------	--SG-L----	------ST-	1b
% tet	**0,04**	**0,1**	< 0,03	< 0,03	
% CD8/IFNy	< 0,03	< 0,03	< 0,03	< 0,03	