Abschlussbericht Teilprojekt 10.2.2

Projekttitel: Therapie der akuten und chronischen Hepatitis C: Immunologische Mechanismen der viralen Clearance

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I. Kurze Darstellung

1, Aufgabenstellung

Ziel des Projektes war die Analyse verschiedener Komponenten zellulärer Immunantworten bei Patienten mit akuter und chronischer Hepatitis C.

2, Voraussetzungen

Das Projekt wurde primär in der AG zelluläre Immunologie (PD Dr. H. Wedemeyer, Medizinische Hochschule Hannover) durchgeführt. Die Infrastruktur zur Durchführung der Experimente wat etabliert. Hep-Net hat das Projekt mit einer BAT 5b-Stelle und Sachmitteln unterstützt. Essentiell war die enge Kooperation mit dem Hep-Net Study, hier im Besonderen die Studie zur akuten Hepatitis C. In dieser Studie wurden Blutproben gewonnen, die dann im Projekt 10.2.2 untersucht wurden.

3, Planung und Ablauf des Vorhabens

Sind detailliert im Antrag sowie in den Zwischenberichten beschrieben.
Es wurden folgende Aspekte zellulärer Immunantworten bei Patienten mit akuter und chronischer Hepatitis C im Kontext antiviraler Therapien mit Interferon untersucht:

- Bedeutung myeloider dendritischer Zellen
- Natürliche Killerzellen
- HCV-spezifische T-Zellantworten
    - Induktion HCV-spezifischer T-Zellen nach Nadelstichverletzung
    - Zytotoxische CD4+ T-Zellen bei viralen Hepatitiden
    - HCV-spezifische T-Zellen bei Patienten mit akuter Hepatitis C
    - HCV-spezifische T-Zellen bei nach Langzeit-Ausheilung einer akuten Hepatitis C

Die Ergebnisse sind auf wissenschaftlichen Tagungen vorgestellt worden und wurden in Fachzeitschriften publiziert.

4, wissenschaftlicher und technischer Stand

Es wurden methodisch hochaktuelle immunologische und molekularbiologische Techniken angewendet, die entweder bereits in der Abteilung für Gastroenterologie, Hepatologie und Endokrinologie der medizinischen Hochschule Hannover etabliert

etabliert waren oder für das Projekt etabliert wurden. Die notwendigen Substanzen (Antikörper, Peptide, Tetramere, molekularbiologische Reagenzien) wurden kommerziell erworben. Schutzrechte bestehen nicht.

1. Angabe der verwendeten Fachliteratur sowie der benutzten Informations- und Dokumentationsdienste
   Literatursuche wurde mittels Medline/PubMed durchgeführt. Bei der verwendeten Fachliteratur handelte es sich um Zeitschriften der Universitätsbibliotheken der Medizinischen Hochschule Hannover.

5, Zusammenarbeit mit anderen Stellen

Alle weiteren Projekte des Bereiches 10.2 (insbesondere Projekt 10.2.1 und 10.2.3), das Hep-Net Start-up Fund Projekt 15.1 sowie das Hep-net Study House.

II. Eingehende Darstellung

1, des erzielten Ergebnisses

Die Darstellung der Ergebnisse erfolgt auf Englisch, da internationale Mitarbeiter im Labor an der Generierung der Ergebnisse entscheidenden Anteil hatten. Diese Mitarbeiter sprechen kein Deutsch. Auf Anfrage können deutsche Zusammenfassungen gerne nachgereicht werden.

1. Dendritic cells

Dendritic cells (DCs) play a central role in antiviral immunity. There are two main populations of DC: myeloid DC (mDC) and plasmacytoid DC (pDC). Most studies have been performed on in vitro derived DC while there are only few data available on freshly isolated DC in viral hepatitis. While the Munich group (Project 10.2.1, Pape/Gruener) has focused on pDC, our project has investigated mDC. The frequency of mDC was found to be lower in patients with chronic hepatitis C than in healthy subjects or recovered patients (chronic hepatitis C vs. controls or recovered patients $p<0.001$). The allostimulatory function of mDC was also significantly weaker in patients with chronic hepatitis C (chronic hepatitis C vs. controls or recovered patients $p<0.001$). In contrast, co-stimulatory molecules such as CD80 and CD86 or

maturation markers such as CD83 were not altered in HCV patients. Similarly, the phagocytotic activity was not impaired in chronic HCV.

We then focussed on a widely unrecognized new effector function of mDC, the direct cytotoxic activity. It has been suggested that measles virus and HIV may enhance cytotoxicity of DCs potentially leading to apoptosis of activated T cells and subsequent down-regulation of antiviral immune responses. We demonstrate that freshly-isolated CD1c-positive myeloid DCs but not BDCA-4-positive plasmacytoid DCs are able to kill target cells mainly via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This cytotoxicity was found to be significantly impaired in patients chronically infected with the hepatitis C virus (HCV) ($p<0.0001$). In contrast, CD1c-positive DCs of patients with a non-viral chronic inflammatory liver disease, primary biliary cirrhosis, displayed normal cytotoxic function. In vitro stimulation with interferon alpha did not alter the cytotoxic activity of CD1c-positive DCs, however, successful antiviral interferon-based therapy of chronic hepatitis C rescued the apoptosis-inducing function of DCs. Thus, in contrast to HIV and measles virus studies on monocyte-derived DCs, freshly isolated myeloid DCs of patients with replicative hepatitis C did not show an increased but a completely abolished ability to lyse target cells when being tested directly ex vivo. The failure of mDC to kill other cells might influence antigen uptake of DCs and could contribute to the increased frequency of autoreactive T cells and autoimmunity in chronic hepatitis C since autoreactive T cells are deleted by dendritic cells.

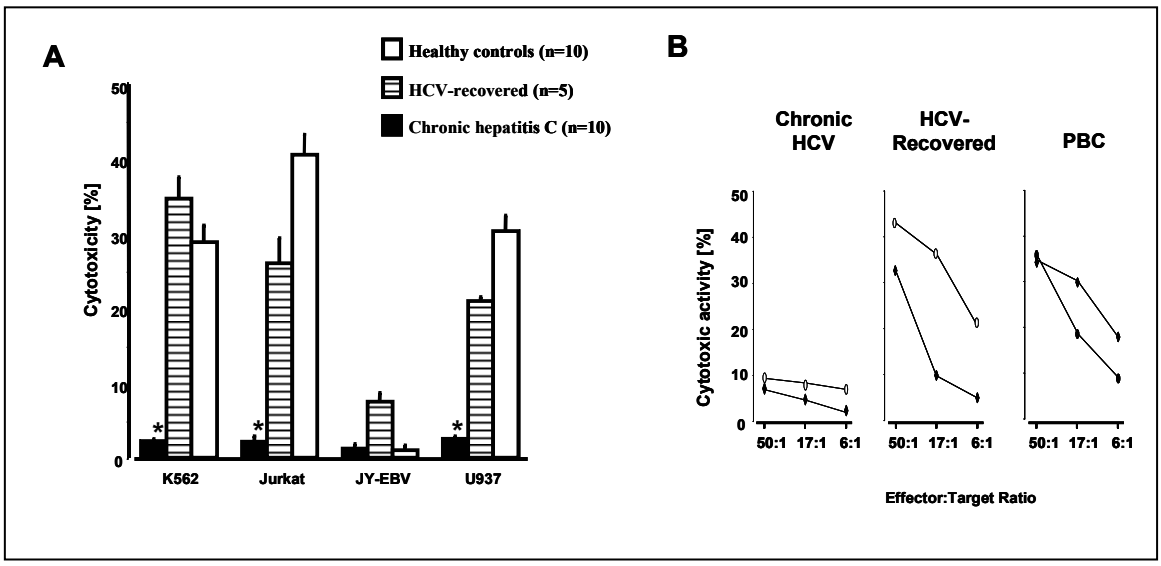

Fig. 1.: Direct ex vivo analysis of mDC cytotoxic activity.

Results of this project are currently in revision in the Journal "Hepatology".

2. Natural Killer cells

Type I interferons have been shown to upregulate tumor necrosis factor-related apoptosis inducing ligand (TRAIL) expression and to augment cytotoxic activity of T cells and natural killer cells in vitro. The aim of this study was to investigate interferon-induced regulation of TRAIL in vitro and in vivo in patients with chronic hepatitis C infection treated with interferon alpha (IFN) and ribavirin. TRAIL-expression was barely detectable on PBMC of chronically infected and long-term recovered individuals (<0,5% positive cells). However, 20 hours of in vitro stimulation with type I interferons but not with ribavirin induced a marked upregulation of TRAIL expression on PBMC (6-42% TRAIL-positive cells) which was significantly higher on PBMC of HCV-patients who have demonstrated a virological response to a previous IFN-alpha-based therapy than on PBMC of IFN-nonresponding patients ($p<0.002$). TRAIL expression was further investigated directly ex vivo during IFN treatment. On day 1 of therapy, TRAIL was expressed on NK-cells but not on T cells (Fig. 2). Expression levels peaked on day 3 and decreased thereafter but remained above baseline levels throughout treatment. The decline of HCV-RNA in serum correlated with increased TRAIL expression on NK cells. In conclusion, type I interferon-induced TRAIL expression on NK cells in vivo correlates with early viral clearance during interferon-alpha therapy of chronic hepatitis C infection. Our data support a potential role for soluble TRAIL as a new therapeutic option not only for cancers but also for viral infections, in particular hepatitis C virus infection.

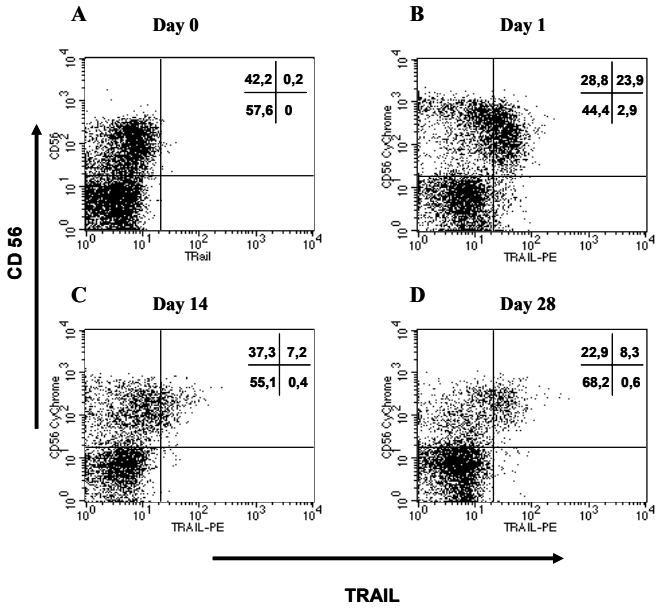

Fig. 2.: Direct ex vivo analysis of TRAIL expression on CD56-positive NK cells during IFN-therapy.

Manuscript submitted.

3. HCV-specific T cells

a. Induction of hepatitis C virus specific T cells by needle stick injury in the absence of HCV-viremia

HCV-specific T cells have been described in HCV-seronegative virus-exposed individuals such as i.v.-drug addicts, family members of chronic HCV-patients and lab-personnel. However, it is not known whether these individuals had recovered from previous asymptomatic acute hepatitis C. We followed 10 individuals who experienced an injury with an HCV-contaminated needle. Between January 2001 and March 2003 we collected PBMC from 10 individuals (medical health professionals at our institution; 4 females, 6 males; age 30-45 years) who experienced an injury with an HCV contaminated needle. Blood samples were taken on the day or the day after the event and at different time points during follow-up for up to 12 months.

None of the individuals became positive for HCV-RNA in serum (TMA-assay) or PBMC and all of them remained anti-HCV-negative throughout follow-up. At the time of the needle stick injury, HCV-specific CD4+ T cell responses were already detectable in 2 of the individuals and became detectable thereafter in 2 additional persons. HCV-specific CD8+ T cell responses could be investigated in one HLA-A2-positive individual in more detail at several time points. He was negative for HCV-specific interferon gamma- and IL-10-producing cells on the day of the injury as determined by ELISPOT assays. However, after 18 weeks, we detected for Core-178-specific IFN-gamma producing CD8+ T cells. Out of 7 MHC-class I-restricted HCV epitopes tested, one additional peptide (NS3-1406) became positive 8 weeks later. The presence of Core-178 and NS3-1406-specific CD8+ T cells was confirmed by a second flow-cytometry-based assay (Figure 3). HCV-specific INF-gamma positive cells were CD8-dim and HLA-DR-negative. The frequency of NS3-1406 and Core-178-specific CD8+ T cells was about 1/4500 and 1/8500 PBMC, respectively, in the ELISPOT assay and remained constant in this subject until month 11 of follow-up. The increase of CD8+ T cell responses was accompanied by the development of an HCV-specific CD4+ response targeting predominantly the HCV-core and HCV-helicase antigens.

Thus, We were able to demonstrate for the first time in a prospective study the development of HCV-specific T cells in HCV-exposed individuals after needle stick injury in the absence of viremia. Our data are in line with previous studies

demonstrating HCV-specific T cell responses in chimpanzees inoculated with sub-infectious doses of HCV. T cell immunity in medical health professionals against HCV may contribute to the low prevalence of HCV among doctors and nurses since HCV prevalence in medical health personnel has been shown to be equal or even lower as compared to the general population in most studies.

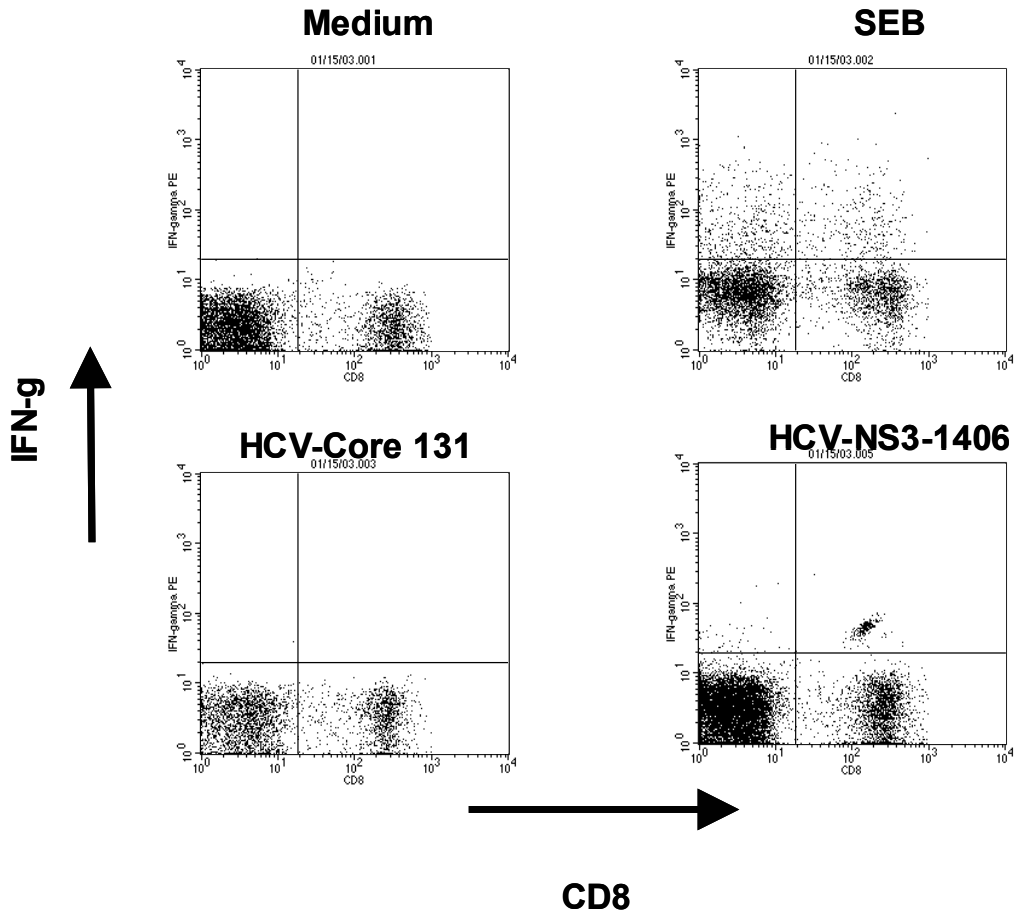

Fig. 3: Detection of HCV-NS3-1406-specific CD8+ T cells by cytokine secretion assay in a subject 47 weeks after injury with an HCV-contaminated needle.

Manuscript submitted.

b. Cytotoxic CD4+ T cells in viral hepatitis

CD4-positive T cells have been shown to be of importance for the control of HBV and HCV infections. Their main function is to secrete cytokines and thereby to provide

help to cytotoxic CD8-positive T cells. However, recently a high frequency of perforin-positive cytotoxic CD4+ T cells has been found in HIV-positive patients (J Immunol 2002;168:5954-8). Thus, we investigated the frequency and function of cytotoxic CD4+ T cells in patients with viral hepatitis. 95 individuals were studied. 43 patients suffered from chronic HBV infection, 18 of those were co-infected with HDV. 29 patients had chronic hepatitis C and 23 were HIV-positive. Intracellular perforin expression of CD4-positive T cells, CD8-positive T cells and CD4/CD8-negative lymphoid cells was investigated by flow cytometry. The frequency of perforin-positive CD4+ T cells was highly variable ranging from less than 1% to 40%. The mean percentage of CD4-perforin-+ cells was 2.4±2.8% (HCV), 3,1±4.2% (HBV without delta), 7.1±7% (HDV, p=0.002 vs. HCV; p=0.03 vs. HBV without delta) and 7.5±11.8% (HIV; p=0.009 vs. viral hepatitis without delta). The frequency of cytotoxic CD4+ T cells did correlate with AST levels, perforin-positive CD8+ T cells and age. Functionality of perforin-positive CD4+ T cells was shown using granzyme-B ELISPOT assays. Interestingly, Perforin-positive CD4+ CTLs declined during spontaneous clearance of acute hepatitis C in two subjects that were followed prospectively.

Thus, we were able to demonstrate the existence of cytotoxic CD4+ T cells in patients with viral hepatitis. However, frequencies in HBV and HCV infections are lower than in HIV infection. The increasing frequency of CD4+ CTLs with age might be one correlate of more advanced disease progression of HCV in elderly individuals.

Manuscript submitted.

c. HCV-specific T cells in patients with acute hepatitis C

The main initial task of the project was to acquire and to cryopreserve PBMC of patients with acute and chronic hepatitis C prior to, during and after antiviral therapy. These cells will be thawed after the end of follow-up and will be tested in one assay for antigen-specific and innate cellular immune responses. By this approach we can minimize the problem of inter-assay variability which is usually significant in the immunological readouts used in T cell projects.

We were able to collect PBMC of >20 patients (!) with newly diagnosed acute hepatitis C infection treated with interferon alpha in the acute HCV treatment trial. Prospective screening for cellular immune responses will be performed when all

patients will have completed therapy. HCV-specific tetramers have been purchased and were used together with Dr. Grüner, project 10.2.1.

In addition, we performed a cross-sectional study of 31 patients who had been treated in the first acute hepatitis C treatment trial between 1998 and 2001 (Jäckel, Cornberg, Wedemeyer et al., N Engl J Med 2001; 345: 1452-7). Median follow-up of these patients was 132 weeks (48-208 weeks) after the end of therapy. Clinical, virological and immunological data were analysed in cooperation with other Hep-net members This project was only possible by the close cooperation with several Hep-Net partners who re-recruited the patients or provided other support. E.g., Dr. Hinrichsen (Kiel) performed the highly sensitive TMA-assay to detect low levels of residual HCV. Results can be summarized as follows:

None of the individuals had clinical evidence of liver disease at their most recent visit. ALT levels were normal in all but one patient. HCV-RNA was negative in serum throughout follow-up, even when investigated with the highly sensitive TMA assay (cutoff 5-10 IU/ml). In addition, no HCV-RNA was detected in PBMC of 15 cases tested. The overall quality of life scores of the study cohort as determined by the SF36 questionnaire did not differ from the German reference control cohort.

Ex vivo interferon-gamma ELISPOT analysis of the cellular immune response detected HCV-specific CD4+ T-helper cell reactivity in only 35 % of cases, whereas HCV-specific CD8+ T-cell responses was found in four out of five HLA-A2-positive individuals tested. Anti-HCV antibody levels decreased significantly over time during and after therapy in all individuals (Figure 4).

In conclusion, early treatment of symptomatic acute hepatitis C with interferon alfa-2b leads to a long-term sustained virological and biochemical response accompanied by normal quality of life in most of the patients. There is no evidence for persisting low levels of viremia after initial clearance of HCV. Weaning of anti-HCV humoral immunity and presence of HCV-specific CD8+ but not of CD4+ T cells in the absence of detectable persisting antigen highlights the complexity of T cell and B cell memory to HCV which might have important implications for the development of vaccines against hepatitis C.

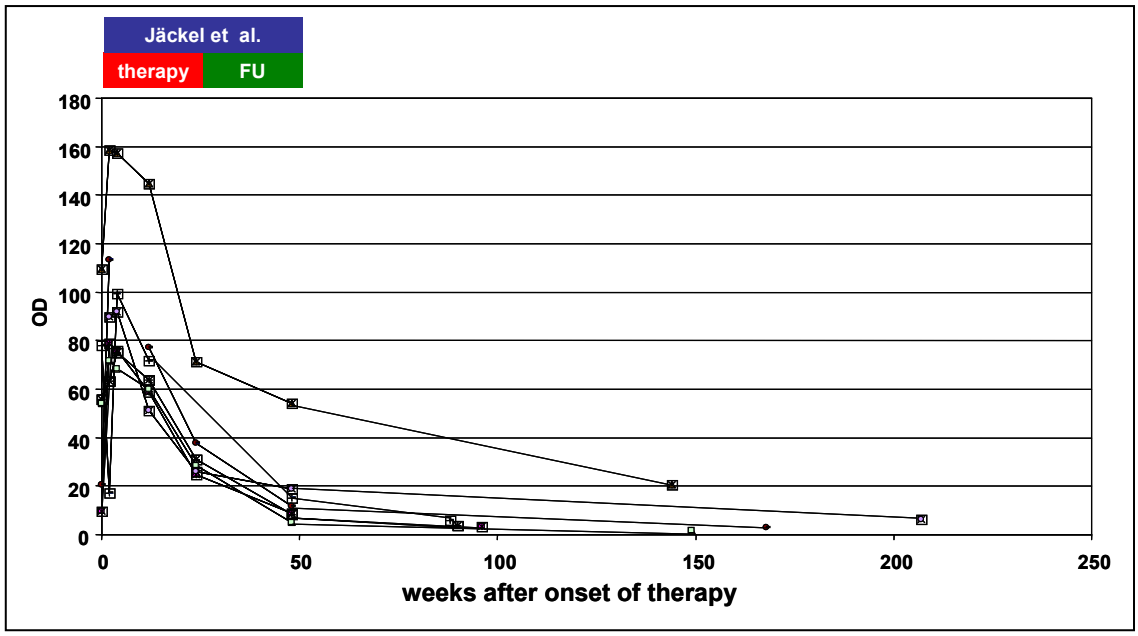

Fig 4: Anti-HCV-antibody levels during and after therapy of acute hepatitis C (Wiegand et al. EASL 2003).

Analysis of T cell responses during PEG-IFN and Ribavirin therapy (Acute HCV-II Study)

The antiviral but also antiproliferative drug interferon-alfa may, however, differentially alter virus-specific T cells in the acute phase of the infection. There are only very few data on HCV-specific T cells during antiviral therapy of acute hepatitis C. We therefore performed a comprehensive analysis of HCV-specific CD4+ and CD8+ T cell responses in eight HLA-A2 positive patients (3 male, 5 female, age 34 ± 13 years, 6 patients with HCV-genotype 1 and 2 with genotype 3) with acute HCV infection who were treated with PEG-interferon alfa-2b for 24 weeks. PBMC were collected frequently prior, during and after therapy and cyropreserved cells were used for interferon-gamma-ELISPOT-assays, proliferation assays and flow-cytometry (HLA-A2-restricted tetramers).

A sustained virologic response was observed in four cases, one patient experienced a relapse and one individual showed a breakthrough. Two additional

patients cleared HCV-RNA during treatment but final follow-up results are pending. T-cell responses were multispecific with different magnitudes before therapy. The only patient with no detectable HCV-specific T cells prior to therapy was the individual who experienced a breakthrough. Both CD4+ and CD8+ T-cells were boostered after initiation of therapy until week 4, but declined thereafter in all individuals and became undetectable in 2 cases. A strong T cell response prior to or during therapy was no prerequisite for successful treatment outcome since two sustained responders demonstrated only weak response in all phases. HCV-specific CD8+ T cells displayed the phenotype of none-terminally differentiated memory cells (CD45RO+, CD27+) and were functional with respect to interferon-gamma production prior to and during therapy. In one individual, functionally impaired cells became detectable during HCV relapse. Interferon therapy significantly influenced maturation of none-HCV specific CD4+ and CD8+ cells as determined by a significant decrease of CD45RO+CD27- cells during therapy.

Thus, kinetics of CD4 and CD8 T-cell responses during interferon therapy are heterogeneous and may not necessarily correlate with treatment outcome. In contrast to very recent published data, successful interferon therapy did not lead to restoration of HCV-specific cellular immunity. Importantly, the general T cell pool is significantly altered by interferon therapy which might have significant consequences for subsequent contact with other infectious agents.

Manuscript published in Hepatology 2004 (Wiegand et al.)
2nd manuscript currently in preparation.

2, des voraussichtlichen Nutzens, insbesondere der Verwertbarkeit des Ergebnisses im Sinne des fortgeschriebenen Verwertungsplanes

Die Ergebnisse zeigen grundlegende neue Erkenntnisse zur Regulation von Immunantworten bei der Hepatitis C durch antivirale Therapien auf. Diese werden bei der Entwicklung von neuen Immuntherapien Berücksichtigung finden. Die Ergebnisse sind auf wissenschaftlichen Kongressen und in hochrangigen wissenschaftlichen Journalen veröffentlicht und damit der wissenschaftlichen Öffentlichkeit zugänglich gemacht worden. Insbesondere werde die Daten für das direkte Management von

Patienten mit akuter Hepatitis C, die mit Interferon behandelt werden, eine große Relevanz haben.

3, des während der Durchführung des Vorhabens dem ZE bekannt gewordenen Fortschritts auf dem Gebiet bei anderen Stellen

Mehrere andere Arbeitsgruppen weltweit beschäftigen sich mit ähnlichen Projekten. Insbesondere die Frage, ob zelluläre Immunantworten während einer IFN Therapie der akuten Hepatitis ansteigen oder abfallen wird sehr kontrovers diksutiert (Kamal et al., Hepatology 2004: Anstieg; Rahman et al., Hepatology 2004: Abfall). Unsere Arbeit konnte in diesem Zusammenhang die Daten von Rahman et al. bestätigen und diese kontroverse Frage klären.

4, der erfolgten und geplanten Veröffentlichung des Ergebnisses

Orginalarbeiten:

1. Potthoff A, Sarhaddar J, Wiegand J, Lichtinghagen R, Sarrazin C, Ciner A, Hadem J, Trautwein C, Manns MP, **Wedemeyer H**. Spontaneous resolution of chronic hepatitis C virus infection after Antiviral Treatment and Relapse. Hepatology Research; *in press*
2. Ciesek S, Ringe BP, Strassburg CP, Klempnauer J, Manns MP, Becker T and **Wedemeyer H**. In vitro and in vivo investigations on the effects of cyclosporine on human dendritic cell subsets. Transplant Proceedings; *in press*
3. Wiegand J, Jäckel E, Cornberg M, Hinrichsen H, Dietrich M, Kröger J, Fritsch WP, Kubitschke A, Aslan N, Tillmann HL, Manns MP, **Wedemeyer H**. Long-term follow up after successful interferon therapy of acute hepatitis C virus infection. Hepatology 2004; 40:98-107.
4. Lehmann M, Meyer M, Monazahian M, Tillmann HL, Manns MP, **Wedemeyer H**.
   High rate of spontaneous clearance of acute hepatitis C genotype 3 infection. J Med Virol 2004; 73: 387-391.

5. **Wedemeyer H**, Cornberg M, Tegtmeyer B, Frank H, Tillmann HL, Manns MP. Anti-HBc alone antibody phenotype in anti-HCV-positive patients is associated with HCV replication. Clin Microbiol Infect 2004; 10: 70-72.

6. Cornberg M, Hüppe D, Wiegand J, Felten G, **Wedemeyer H**, Manns MP. Therapie chronischen Hepatitis C mit PEG-Interferon alfa-2b und Ribavirin: 24 Wochen sind ausreichend bei den HCV-Genotypen 2 und 3. Z Gastroenterol 2003; 41: 517-522.

Abstracts:

1. Aslan N, Yurdaydin C, Wiegand J, Tillmann HL, Kaiser T, Kulmann B, Bozkaya H, Meyer M, Bozdayi AM, Manns MP, **Wedemeyer H**. Cytotoxic CD4-positive T cells in viral hepatitis. J Hepatol 2004; 40 Suppl. 1: 86.

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4. Kubitschke A, Bahr M, Aslan N, Sarrazin C, Tillmann HL, Greten T, Meyer M, Wiegand J, Manns MP, **Wedemeyer H**. Induction of hepatitis C virus specific T cells by needle stick injury in the absence of HCV-viremia. Hepatology 2003; 38: Suppl. 1, 172A.

5. Meyer MF, **Wedemeyer H**, Dreesman J, Manns MP, Baillot A, Lehmann M. Hepatitis C Virus infection in a juvenile prison: High prevalence of HCV among immigrants from the former Soviet Union to Germany. Hepatology 2003; 38: Suppl. 1, 465A.

6. Wiegand J, Jäckel E, Cornberg E, Dietrich M, Hinrichsen H, Kröger J, Fritsch WP, Kubitschke A, Aslan N, Tillmann HL, Manns MP, **Wedemeyer H.** Immunological, virological and clinical long-term follow-up after interferon therapy of acute hepatitis C infection. J Hepatol. 2003; 38: Suppl. 2: 181.

7. Ciesek S, Becker T, Strassburg CP, Ringe B, Klempnauer J, Manns MP **Wedemeyer H**. A novel immunosuppressive function of anti-IL2 receptor antibodies. Hepatology 2004; 40: 548A.

8. Wiegand J, Aslan N, Ciner A, Buggisch P, Jäckel E, Cornberg M, Manns MP **Wedemeyer H**. Kinetics of HCV-specific CD4+ and CD8+ T cell responses during and after therapy with pegylated interferon-alfa-2b in patients with acute hepatitis C. Hepatology 2004; 40: 256A.