

Abschlussbericht Teilprojekt 10.2.3

Projekttitle: Dendritic cell-based immunotherapy of chronic HCV infection
(Immuntherapie der chronischen HCV-Infektion mittels dendritischer Zellen)

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I. Aims and summary of the work

- Characterization of the monocyte derived mature and immature DC phenotype from HCV and HBV infected versus healthy persons.
- Cloning and expression analysis of targeting vectors.
- Characterization of the different DC targeting strategies on DC phenotype and maturation.
- Influence of the different DC targeting strategies on CD4+ and CD8+ T cell responses.

The results mentioned below which could be achieved during the study period will enable further studies of immunotherapeutic strategies against chronic HBV and HCV infection. The ongoing studies of Prof. Thimme (Uniklinik Freiburg) will complement and extend our research efforts which ultimately should result in the initiation of clinical trials in chronically HBV or HCV infected patients refractory to IFN-therapy. Significant progress has been achieved characterizing the DC function in HBV and HCV chronically infected patients as well as in the development of new DC-based vaccination strategies. Similar projects are ongoing with respect to chronic HBV infection, both in humans and murine models (see below).

II. Results

1. Development of a single shot full genome DNA vaccine against chronic HBV infection.

Grimm C.F., Weth R., Köck J., Endrulat K., Gaiser S., Böcher W.O. and M. Geißler (zur Publikation eingereicht)

Introduction: The activity of a broad based immune response to HBV antigens has been shown to be one of the most important factors contributing to virus elimination from infected hepatocytes. Our aim was, therefore, to develop a DNA-based vaccine expressing HBV gene products but lacking HBV replication. Therefore, a CMV-promoter based plasmid producing a HBV pregenome lacking the first 43 nucleotides resulting in a mutated epsilon-signal and subsequently a defective HBV pregenome encapsidation (pCH3143) was employed. **Materials and methods:** pCH3143 was transfected in G8 murine myoblast and Huh-7 human hepatoma cells. Expression of HBcAg, HBsAg, HBeAg, polymerase, and x-antigen was determined by Western-blot, immunofluorescence and ELISA techniques. HBV replication and RNA analysis was assessed by Southern blot and Northern blot, respectively. Balb/c and C57BL/6 mice were immunized once with 100 µg pCH3143 and, subsequently, antibody as well as CTL responses against all viral gene products were determined by ELISA and cytotoxicity assays. Protective immunity was determined by re-challenge of pCH3143-immunized Balb/c mice with syngeneic tumor cells expressing HBcAg, HBeAg, the small and large HBsAg, and polymerase. **Results:** After transfection of both G8 and Huh-7 cells high level expression of HBeAg, polymerase, and all HBsAg (small, pre-S2, pre-S1) could be detected. By contrast, expression of x-antigen was weak. No HBV replication was detectable, whereas high level replication was observed in cells transfected with a non-mutated control plasmid. No encapsidation of pregenomic RNA in cores could be observed and the corresponding RNA was detected in 10-20 fold increase amounts compared to wild-type HBV RNAs produced from the HBV wild-type construct. Immunization of Balb/c and C57BL/6 mice resulted in strong anti-HBs and anti-HBc antibody responses. Only low-titer antibodies against polymerase and no anti-HBx could be detected. Strong CTL responses could be observed against all viral antigens in a hierarchical manner in both mouse strains (HBcAg>HBsAg>polymerase>HBxAg). Corresponding to the in vitro CTL activity, pCH3143 vaccinated animals were protected against a s.c. challenge with syngeneic tumors

expressing HBcAg, HBsAg, and polymerase. Mice immunized with Mock-DNA were not protected. Similarly, there was a rapid growth of x-antigen expressing tumors in pCH3143 immunized animals. Discussion: This is the first demonstration of a single shot vaccine expressing all HBV antigens, lacking HBV replication, and inducing strong humoral and cellular immune responses against all viral proteins except x-antigen. This vaccine may be of value for the treatment of chronic viral hepatitis, for vaccine non-responders, and the prophylaxis of HCC.

2. Characterization of immune responses against the hepatitis B virus (HBV) X-protein after DNA-based immunization.

Stefan Meckel, Christian Grimm, Dörte Ortmann, Christian Mauch, Robert Weth, Hubert E. Blum and Michael Geissler. (submitted)

We have previously shown that hepatitis B virus (HBV) surface and core antigens are highly immunogenic after DNA-based immunization. In the present study, we analyzed the immune responses against HBV X-protein in different mouse strains. Only low titer anti-HBx antibodies could be observed in C57BL/6 and Balb/c mice after immunizations with HBx-antigen expressing plasmids of both subtype adw and ayw, respectively. Antibody titers could not be augmented by increasing numbers of immunization or cytokine co-immunizations using IL-12 or IL-18 DNA expression plasmids. Moderate T-cell proliferative responses could be demonstrated in both mouse strains. No cytotoxic T cell responses could be observed using vaccinia virus based ELISPOT and chromium release assays. A panel of H-2b and H-2d consensus sequence peptides as well as overlapping 15 mer peptides were further analyzed for reactivity with CD8⁺ T cells derived from immunized animals. Again, no cytotoxic activity could be demonstrated. Together, these results suggest that HBx-antigen seems to be a weak B and T cell immunogen. Due to its weak expression *in vivo* during HBV replication immunotherapeutic strategies against chronic HBV infection using HBx as target may not be promising.

3. Immunoregulation of dendritic and T cells by alphafetoprotein in patients with hepatocellular carcinoma.

Marcus Ritter, Mona Y. Ali, Christian F. Grimm, Robert Weth, Leonhard Mohr, Wulf O. Bocher, Katja Endrulat, Heiner Wedemeyer, Hubert E. Blum, and Michael Geissler *J Hepatol.* 2004 Dec;41(6):999-1007.

Background and Aims: Hepatocellular carcinoma (HCC) is a tumor with a poor prognosis. Therefore, novel immunotherapeutic and other strategies are being explored. Alpha-fetoprotein (AFP) is overexpressed in 60-80% of HCCs and, therefore, may be a target antigen for immunotherapy. Little is known, however, about the immunobiology of AFP. Therefore, the impact of AFP on peripheral blood immune effectors such as dendritic cells (DC), CD4⁺ and CD8⁺ T cells was studied in detail. Materials and Methods: Immune cells derived from peripheral blood of 27 HCC patients with AFP serum concentrations ranging from 0 to 300.000 ng/ml were studied. Monocyte derived (Mo-DC), plasmacytoid (PDC) and myeloid dendritic cells (MDC), as well as CD4⁺ and CD8⁺ T cells were functionally analyzed directly *ex vivo* and *in vitro* using FACS, ELISPOT, and proliferation assays. Results and Discussion: The *in vitro* generation, maturation, and T cell stimulatory capacity of Mo-DC were not altered by AFP up to concentrations of 20 mg/ml. Only higher AFP concentrations (>20 mg/ml) resulted in phenotypic changes on Mo-DCs without impairing their capacity to stimulate CD4⁺ T cells. No significant differences in frequencies and expression of maturation markers of MDC or PDC were observed in HCC patients dependent on serum AFP levels. Allogenic helper T cell stimulatory capacity of MDC and PDC was comparable between HCC patients with normal and elevated AFP serum levels. In addition, AFP specific T cell proliferative response and numbers of IFN- γ and IL-5 producing AFP specific PBMCs, CD4⁺, and CD8⁺ T cells did not significantly differ between HCC patients with normal and high serum AFP levels, respectively. Finally, T lymphocytic infiltrations in the liver were not dependent on AFP serum levels. These studies clearly demonstrate that i) DC-based immunotherapeutic approaches are a promising approach for HCC treatment and ii) AFP-

reactive T cell clones have not been deleted from the human T cell repertoire independent of the level of AFP re-expression by the tumor in the majority of HCC patients (>80%) establishing AFP as a potential target for T cell based immunotherapy of HCC.

4. Phenotype and function of dendritic cells in HCV chronically infected patients

C. F. Grimm, K. Endrulat, M. Ritter, W.O. Böcher, H. Wedemeyer, H. E. Blum, M. Geissler (*manuscript in preparation*).

To better understand the function of DC in HCV infected patients we initially started to analyze the function of monocyte derived DC (Mo-DC). We were, however, not able to find significant differences in Mo-DC function between healthy controls (n=5) and chronically HCV infected patients (n=9). Expression of CD80, CD83, CD86, CD40, and HLA-DR, FITC-dextran uptake, and allogeneic CD4⁺ T cell stimulation were not different between both groups. Since Mo-DC do not reflect the *in vivo* situation due to extensive *in vitro* generation steps, we started to analyze the two major peripheral blood DC subsets (CD1c⁺ CD11c⁺ myeloid DC (MDC) and CD123⁺ CD11c⁻ plasmacytoid DC (PDC)) in HCV infected patients directly *ex vivo*. This project was planned and performed in close collaboration with Dr. Böcher, Mainz and Dr. Wedemeyer, Hannover.

Shortly, PBMCs were depleted of CD19⁺ cells. Then, MDC and PDC were isolated by indirect magnetic labeling, followed by enrichment of labelled cells using two cycles of an immunomagnetic selection device. Finally, CD8⁺ and CD4⁺ cells were isolated using anti-CD8- and anti-CD4-Mircobeads, respectively. Purity of each isolated cell type was >95 % . The remaining cells were used for *in vitro* generation of Mo-DC.

We subsequently analyzed the phenotype and functions of MDC and PDC in 5 healthy volunteers and 17 chronically HVC infected patients directly *ex vivo*. Cells were quantified by multiparametric flow cytometry of whole blood. The median frequency of MDC in control individuals was 0.31%, whereas that of PDC was 0.29%. Pooled frequencies of MDC (0.33±0.1%) and PDC (0.31±0.08%) derived from all HCV patients were comparable to those in healthy volunteers. MDC and PDC isolated from HCV patients were further studied by FACScan for the expression of DC differentiation markers (CD83, HLA-DR) and costimulatory molecules (CD86, CD80, CD40). In all patients and controls, MDC and PDC derived from peripheral blood displayed the characteristic phenotype of immature DC: expression of moderate to high levels of MHC class II antigens, low levels of CD86, no or only faint CD83, and no CD80 or CD40. We now assessed the capacity of MDC and PDC to induce PBMC proliferation in an allogenic mixed lymphocyte reaction (MLR). In general, T cell proliferation upon stimulation with MDC or PDC was much weaker compared to stimulation with Mo-DCs. The allostimulatory function of MDC between HCV infected patients and controls was not different (p>0.5). Similarly, the allostimulatory function of PDC was comparable between HCV patients and controls, though significantly weaker than that of MDC. Therefore, our results demonstrate that number and function of MDC and PDC from HCV patients are not impaired.

5. Characterization of the biological activities of uridine diphosphate in human dendritic cells: Influence on chemotaxis and CXCL8 release.

Idzko M, Panther E, Soricter S, Herouy Y, Berod L, Geissler M, Mockenhaupt M, Elsner P, Girolomoni G, Norgauer J. (Manuskript in Vorbereitung)

Uridine nucleotides are endogenous nucleotides which are released into the extracellular space from mechanically stressed endothelial and epithelial cells as well as lipopolysaccharide (LPS)-stimulated monocytes. Here, we studied the biological activity of the selective purinoreceptor P2Y₆ (P2Y_{R6}) agonist Uridine 5'diphosphate (UDP) as well as the P2Y_{R2}- and P2Y_{R4}-activating uridine 5'triphosphate (UTP) on human dendritic cells (DC). These cells in their immature state have the ability to migrate from blood to peripheral target sites where they sense dangerous signals and capture potential antigens. Moreover, mature DC induce innate immune responses and migrate from peripheral tissues to secondary lymphoid

organs in order to activate naive T cells and initiate adaptive immunity. Here, we were able to show that uridine nucleotides stimulated Ca^{2+} transients, actin polymerization, and chemotaxis in immature DC. Experiments with pertussis toxin, the stable pyrimidine agonist uridine 5'-O-(2-thiodiphosphate) (UDP γ S) and receptor antagonists, as well as desensitization studies suggested that these uridine nucleotides activities were mediated by different G(i) protein-coupled receptors. During Ips-induced maturation, DC lost their ability to respond towards uridine nucleotides with these activities. Instead, UDP, but not UTP, stimulated the release of the CXC-chemokine 8 (CXCL8) from mature DC in a reactive blue sensitive manner. Moreover, our study indicates that UDP stimulates different signaling pathways in immature and mature DC in order to favor the accumulation of immature DC and to augment the capacity to secrete CXCL8 in mature DC.

6. The serotonergic receptors of human dendritic cells: identification and coupling to cytokine release.

Idzko M, Panther E, Stratz C, Muller T, Bayer H, Zissel G, Durk T, Sorichter S, Di Virgilio F, Geissler M, Fiebich B, Herouy Y, Elsner P, Norgauer J, Ferrari D. (Manuskript in Vorbereitung)

The neurotransmitter 5-hydroxytryptamine (5-HT), commonly known as serotonin, is stored at peripheral sites in mast cells and released from this peripheral source upon IgE cross-linking. In this study, we investigated the expression of serotonergic receptors (5-HTR), the signaling pathway, and biological activity of 5-HT on human dendritic cells (DC), showing that immature and mature DC expressed mRNA for different serotonergic receptors. Thereby, the mRNA of 5-HTR(1B), 5-HTR(1E), 5-HTR(2A), 5-HTR(2B), one splicing variant of the 5-HTR(3), 5-HTR(4), and 5-HTR(7) receptors were detected. Immature DC preferentially expressed mRNA for the heptahelical 5-HTR(1B), 5-HTR(1E), and 5-HTR(2B) receptors, while mature DC mostly expressed 5-HTR(4) and 5-HTR(7). The mRNA expression level of the ligand-gated cation channel 5-HTR(3) and the heptahelical 5-HTR(2A) did not significantly change during maturation. Isotype-selective receptor agonists allowed us to show that 5-HT stimulated 5-HTR(3)-dependent Ca^{2+} influx in immature and mature DC. Moreover, we revealed that 5-HTR(1) and 5-HTR(2) receptor stimulation induced intracellular Ca^{2+} mobilization via G(i/o) proteins in immature, but not mature, DC. Activation of 5-HTR(4) and 5-HTR(7) induced cAMP elevation in mature DC. Functional studies indicated that activation of 5-HTR(4) and 5-HTR(7) enhanced the release of the cytokines IL-1 β and IL-8, while reducing the secretion of IL-12 and TNF- α in mature DC. In summary, our study shows that 5-HT stimulated, in a maturation-dependent manner, different signaling pathways in DC. These data point to a role for 5-HT in regulating the immune response at peripheral sites.

7. Activation of dendritic cells by local ablation of hepatocellular carcinoma.

Ali MY, Grimm CF, Ritter M, Mohr L, Allgaier HP, Weth R, Bocher WO, Endrulat K, Blum HE, Geissler M. *J Hepatol.* 2005 Nov;43(5):817-22.

BACKGROUND/AIMS: Local ablation methods are an effective treatment for hepatocellular carcinoma (HCC). The rate of recurrence or development of intra-hepatic metastases may be lowered by antitumoral immune responses. Since HCCs are in general only weakly immunogenic, cell injury induced by local tumor ablation (PEI/RFTA) may increase HCC immunogenicity and may release endogenous adjuvants that activate dendritic cells (DC). The aim of the study, therefore, was the analysis whether PEI or RFTA induced injury results in an adjuvant effect for immune responses to HCCs. **METHODS:** Eight HCC patients were treated with PEI or RFTA and serially analyzed for 4 weeks. Plasmacytoid (PDC) and myeloid dendritic cells (MDC) were analyzed directly ex vivo and in vitro using FACS and proliferation assays. **RESULTS:** HCC ablation induced a functional transient activation of MDC but not of PDC associated with increased serum levels of TNF- α and IL-1 β . **CONCLUSIONS:** These findings suggest that the combination of PEI or RFTA and active antigen specific immunotherapeutic approaches using DCs is a promising approach for the

induction of sustained antitumoral immune responses aiming at the reduction of tumor recurrence and metastases in HCC patients.

8. Intrahepatic accumulation of AFP-specific T cell responses in patients with HCC ± HCV infection.

R. Thimme, M. Neagu, N. Kersting, C. Neumann-Haefelin, H.E. Blum, and H.C. Spangenberg (Manuskript in Vorbereitung)

It was the aim of this study to analyze the vigour and breadth of AFP T cell responses in patients with chronic HCV infection with and without hepatocellular carcinoma (HCC) and to compare it with the virus-specific T cell responses. In order to determine the CD8(+)-T-cell responses in the context of diverse HLA class I alleles, we performed a comprehensive analysis by using a panel of overlapping peptides spanning the AFP protein and whole HCV genome and analyzed the peripheral blood mononuclear cells and intrahepatic lymphocytes from a cohort of 11 anti-HCV-positive individuals, 5 anti-HCV-positive HCC patients and 4 anti-HCV-negative HCC patients and PBMCs from 10 healthy individuals by an enzyme-linked immunospot assay followed by peptide specific intracellular interferon- γ staining. The results can be summarized as follows: (1) In HCC patients, AFP specific CD8+ T cell responses were significantly enriched in the liver in comparison to the peripheral blood (38 peptide responses versus 19 responses) suggesting a tumor and site specific immune response. (2) AFP specific CD8(+)-T-cell-responses were also detectable in the peripheral blood and the liver of patients with chronic HCV infection. However, these responses were less vigorous and not enriched in the intrahepatic compartment. (3) The newly identified AFP-peptide specific responses are mainly located at the N-terminus of the protein and are different from previously described immunodominant AFP-specific T cell responses. (4) HCV specific CD8+ T cell responses were only detectable in patients with chronic infection and were then significantly enriched in the liver.

In conclusion, we were able to identify various new AFP-specific T cell responses, that are present in patients with HCC but also in patients with chronic HCV infection. Importantly, however, a compartmentalization of these AFP specific CD8+ T cell responses into the liver was only observed in patients with HCC, supporting a tumor specific accumulation.