Abschlussbericht Teilprojekt 10.2.3

Projekttitel: Immuntherapie der chronischen HCV-Infection basierend auf dendritischen 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.

During the last 3 years, significant progress has been achieved with respect to the characterization of DC function in HBV and HCV chronically infected patients as well as in the development of new DC-based vaccination strategies. Besides targeting HCV, similar projects are ongoing with respect to chronic HBV infection, both in humans and murine models (see also presentations, publications). They are also performed in close collaboration with Dr. Böcher (Mainz), Dr. Wedemeyer (Hannover), and Dr. Diepolder (München) using the Hep-Net network.

II. Results

II.1 Studies aiming at the characterization and optimization of liver specific B and T cellular immune responses

Targeting murine immune responses to selected T cell- or antibody- defined determinants of the hepatitis B surface antigen by plasmid DNA vaccines encoding chimeric antigen.

Schirmbeck, R., X. Zheng, M. Roggendorf, M. Geissler, F. V. Chisari, J. Reimann, and M. Lu. 2001. *J.Immunol.* 166:1405-1413.

By exchanging sequences from the middle-surface (MS) and small-surface (S) Ag of hepatitis B virus (HBV) with corresponding sequences of the MS Ag of woodchuck hepatitis virus, we constructed chimeric MS variants. Using these constructs as DNA vaccines in mice, we selectively primed highly specific (non-cross-reactive) Ab responses to pre-S2 of the HBV MS Ag and the "a" determinant of the HBV S Ag, as well as L^d- or K^b-restricted CTL responses to HBV S epitopes. In transgenic mice that

constitutively express large amounts of HBV surface Ag in the liver we could successfully suppress serum antigenemia (but not Ag production in the liver) by adoptive transfer of anti-pre-S2 or anti-"a" immunity but not CTL immunity. DNA vaccines greatly facilitate construction of chimeric fusion Ags that efficiently prime specific, high-affinity Ab and CTL responses. Such vaccines, in which sequences of an Ag of interest are exchanged between different but related viruses, are interesting tools for focusing humoral or cellular immunity on selected antigenic determinants and elucidating their biological role.

Immunotherapy directed against α-fetoprotein results in autoimmune disease during liver regeneration in mice.

Geissler M., Mohr L., Ortmann D., Grimm C., Krohne T.U., Blum H.E. 2001. Gastroenterology, 121:931-939.

Background and Aims: Priming immune responses against α-fetoprotein (AFP) highly expressed in the majority of hepatocellular carcinomas (HCC) results in significant anti-tumoral T cell responses. Liver regeneration in humans and mice, however, is also associated with increased AFP expression. Therefore, we evaluated the risk of AFP directed immunotherapeutic approaches to induce autoimmunity against the regenerating liver. Methods: Mice were immunized with DNA encoding mouse AFP. For induction of liver regeneration partial hepatectomy was performed and mice were monitored by serial histopathologic examinations and measurements of serum ALT activities (U/l), and by determination of the kinetics of AFP specific T cell responses. Results: Livers of AFP immune mice without partial hepatectomy were characterized by minor lymphocytic infiltrations without transaminase elevations. By contrast, a significant hepatocyte damage was observed in regenerating liver that correlated well with the number of AFP specific CD8+ T cells, the activity of liver regeneration and the level of AFP synthesis. Autoimmune liver damage was mediated by CD4+ T cell dependent CD8+ CTL. Discussion: These results demonstrate that priming of T cell responses against shared tumor specific self antigens may be accompanied by induction of autoimmunity dependent on the level of expression of the self antigen and have important implications for the development of anti-tumoral vaccines targeted against antigens that are not strictly tumor-specific.

II.2 Vaccination studies

Induction of cytotoxic T lymphocyte responses against hepatitis delta virus antigens which protect against tumor formation in mice.

Mauch C., Grimm C., Wands J.R., Blum H.E., Roggendorf M., Geissler M. 2002. Vaccine, 20: 170-180.

The cellular immune response is a crucial defense machanism against hepatotropic viruses and in chronic viral hepatitis prevention. Moreover, hepatitis D (HDV) immunogenicity may be an important component in the development of prophylactic and therapeutic vaccines. Therefore, we evaluated the immunogenicity of the small (HDAg) or large delta antigen (LHDAg) to be used as a DNA-based vaccine. We immunized different mouse haplotypes, determined cellular immune responses, and tested protection of animals against tumor formation using syngeneic tumor cells stably expressing the delta antigens. Both LHDAg and HDAg primed CD4+ and CD8+ T cell immunity against both forms of delta antigens. CD8+ T cell frequencies were about 1% and antigen specific CD8+ T cells remained detectable directly ex vivo for at least 35 days postinjection. No anti-delta antibody responses could be detected despite multiple detection systems and varied immunization approaches. We observed protection against syngeneic tumor formation and growth in mice immunized with DNA plasmids encoding secreted or intracellular forms of HDAg and LHDAg but not with recombinant HDAg establishing the generation of significant cellular immunity in vivo. Both CD4+ and CD8+ T cells were required for antitumoral activity as determined by in vivo T cell depletion experiments. The results indicate that DNA-based immunization with genes encoding LHDAg and HDAg induces strong T cell responses and, therefore, is an attractive approach for the construction of therapeutic and prophylactic T cell vaccines against HDV.

Induction of strong hepatitis B virus (HBV) specific T helper cell and cytotoxic T lymphocyte responses by therapeutic vaccination in the Trimera mouse model of chronic HBV infection. Böcher W.O., Dekel B., Schwerin W., Geissler M., Hoffmann S., Arditti F., Cooper A., Bernhard H. Berrebi A., Rose-John S., Shaul Y., Galle P.R., Löhr H.F., Reisner Y. 2001. Eur. J. Immunol. 31:2071-2079.

Humanized Balb/c mice (termed trimera mice) conditioned by lethal total body irradiation and bone marrow transplantation from SCID mice have been described to support rapid engraftment of human peripheral blood mononuclear cells (PBMC) and the induction of strong B and T cell responses after immunization in vivo. However, like in hu-PBL-SCID mice primary responses against neo-antigens were weak or absent in the trimera. Earlier studies gave evidence for a failure of Th cell priming in the trimera mice. In the current study, the induction of strong primary Th cell responses against a viral and a bacterial neo-antigen (i.e. HBV core (HBc) and Borrelia Burgdorferi (BB)) were induced by transfer of antigen loaded dendritic cells (DC) together with autologous PBMC. Moreover, vaccination with high doses of these antigens or DNA plasmids encoding for HBcAg did stimulate equivalent primary antigen specific Th cell responses. Finally, peptide specific CTL responses against HBcAg and an immunodominant EBV epitope were induced by protein, DNA or peptide vaccination in vivo. Thus, since the trimera can be experimentally infected with HBV or HCV, this model will enable studies of new vaccination strategies such as peptide or DNA vaccination against various human infections, such as HBV or HCV infection.

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

Hepatology October 2004. Abstract. (inzwischen 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, HBsAgs, 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 □g 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 HBsAgs, and polymerase. Results: After transfection of both G8 and Huh-7 cells high level expression of HBeAg, polymerase, and all HBsAgs (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.

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. 2003. J. Hepatol. 38: A377. (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 week 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.

II.3 Immunoregulation of dendritic cells in patients with virally induced liver disease

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), plasmocytoid (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 □g/ml. Only higher AFP concentrations (>20 □g/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-α 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.

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 $CD123^+$ $CD11c^-$ plasmocytoid 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.

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

Idzko M, Panther E, Sorichter S, Herouy Y, Berod L, Geissler M, Mockenhaupt M, Elsner P, Girolomoni G, Norgauer J.

Department of Pneumology, University of Freiburg, Germany.

Uridine nucleotides are endogenous nucleotides which are released into the extracellular space from mechanical stressed endothelial and epithelial cells as well as lipopolysaccharide (lps)-stimulated monocytes. Here, we studied the biological activity of the selective purinoreceptor P2Y6 (P2YR6) agonist Uridine 5'diphosphate (UDP) as well as the P2YR2- and P2YR4-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) (UDPgammaS) and receptor antagonists, as well as desensitization studies suggested that these uridine nucleotides activities were mediated by different G(i) protein-coupled receptors. During lps-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.

The serotoninergic 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.

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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 serotoninergic 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 serotoninergic 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-1beta and IL-8, while reducing the secretion of IL-12 and TNF-alpha 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.

These results which could be achieved during the last 3 years are the basis for the further examination of immunotherapeutic strategies against chronic HBV and HCV infection which ultimately should result in the initiation of phase I/II trials in chronically HBV or HCV infected patients refractory to IFN-therapy.

Using the excellent animal models (HBV transgenic mice in Freiburg, Trimera mouse in Mainz), we had an excellent collaboration ongoing with Dr. Böcher regarding the efficacy of the different vaccination modes with respect to the induction of antiviral immunity and suppression of viral replication. There was also a continous exchange of know-how and materials between the immunological groups in Mainz, Hannover, Munich and Freiburg which were mandatory for the success of this project.