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Abstracts
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Falk Workshop Regensburg

Liver and Immunology
January 27 – 28, 2011
Medical University Regensburg (UKR)
Regensburg, Germany
Abstracts of Invited Lectures
Poster Abstracts

Falk Workshop

LIVER AND IMMUNOLOGY

Regensburg (Germany)
January 27 – 28, 2011

Scientific Organization:
H.J. Schlitt, Regensburg (Germany)
V. Benseler, Regensburg (Germany)
C. Hellerbrand, Regensburg (Germany)
M. Loss, Regensburg (Germany)
T. Weiss, Regensburg (Germany)
R. Wiest, Regensburg (Germany)
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Session I

From inflammation to fibrosis
Toll-like receptors and hepatic wound healing responses

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Inflammation is a characteristic feature of chronic liver disease and believed to promote the progression to fibrosis and hepatocellular carcinoma (HCC). Despite the strong association between fibrosis and inflammation, the functional contribution of inflammation to fibrosis and subsequent HCC development is not yet completely understood. Recent evidence suggests that the intestinal microbiota and toll-like receptors (TLRs) promote wound healing responses in the liver, and that TLRs play an important role in the activation of hepatic stellate, the primary fibrogenic cell type of the liver. Hepatic fibrosis is associated with increased levels of LPS in both mouse models of fibrosis and patients with hepatic fibrosis. Genetic inactivation of TLR4, gut sterilization by antibiotics (significantly reducing plasma LPS levels) or treatment with antibiotics ameliorate hepatic fibrosis in different models of experimental fibrogenesis. Moreover, genetic inactivation of TLR4 and treatment with antibiotics also strongly reduce the occurrence of HCC in models in which HCC is driven by chronic injury and inflammation. Conversely, LPS treatment promotes HCC development. Bone marrow transplantation excludes Kupffer cells as mediators of the profibrogenic and carcinogenic effects of TLR4. In vitro and in vivo experiments have revealed that hepatic stellate cells are highly responsive to LPS. Finally, recent studies suggest that single nucleotide polymorphisms that are associated with reduced TLR4 responsiveness decrease the risk for fibrosis in patients with chronic hepatitis C reduce. In conclusion, there is increasing evidence the intestinal microbiota and TLR4 link between inflammation, hepatic stellate cell activation, liver fibrosis and HCC.
Adipokines and fibrosis

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Hepatic fibrosis is a dynamic process whereby the liver responds to a condition of persistent damage. This leads to deposition of fibrillar extracellular matrix, altered hepatocyte regeneration, deranged microvascular architecture and cirrhosis. Accumulating data demonstrate that obesity and insulin resistance are associated with a more severe and faster progression of the fibrogenic process in different chronic liver diseases, and attention has focused on possible links between the adipose tissue and liver repair.

Adipokines are polypeptides secreted in the adipose tissue in a regulated manner. While some of these molecules are expressed only by adipocytes, resident and infiltrating macrophages markedly contribute to expression of other adipokines. As a result, adipose tissue inflammation is associated with a modification in the pattern of adipokine secretion. Leptin, adiponectin and resistin are the best studied molecules in this class, but cytokines such as tumor necrosis factor or interleukin-6 are also secreted at high levels by the adipose tissue. Several other molecules have been recently identified, including chemerin, vaspin, retinol binding protein-4 and apelin, which are being actively investigated. Most of the available data focus on leptin and adiponectin.

Expression of leptin occurs predominantly in the adipose tissue, but is found also at many other sites. Leptin has been extensively characterized for its profibrogenic role, and several cell types contribute to this action. Leptin directly targets hepatic stellate cells (HSC) via activation of ObRb, triggering a downstream cascade that includes ERK1/2, PI3K/Akt, mTOR and HIF-1. Of note, the actions of leptin on HSC also include activation of NADPH oxidase and ROS production, which regulate chemokine expression, and phagocytosis of apoptotic bodies. Leptin has been recently associated with cancer development, both directly and through increased angiogenesis.

Adiponectin binds at least two specific receptors, AdipoR1 and AdipoR2. AdipoR1 is expressed in skeletal muscle and other tissues, while AdipoR2 is mostly expressed in the liver. The main downstream effector of AdipoR1 is AMP-activated protein kinase (AMPK), while AdipoR2 signals via peroxisome proliferator-activated receptor-α (PPAR-α).

Adiponectin reduces inflammation in several models of liver injury, stimulating secretion of anti-inflammatory cytokines (e.g. IL-10), blocking NF-κB activation, and inhibiting release of TNF-α, IL-6, and chemokines. Conversely, inflammation blocks adiponectin secretion. Adiponectin knockout mice develop more extensive fibrosis than wild type animals after chronic CCl4 intoxication, demonstrating that adiponectin has antifibrogenic effects independently of metabolic actions. Reduced fibrogenesis is mediated at least in part by modulation of the activated phenotype of HSC, which express both adiponectin receptors. Activation of AMPK has been identified as a pivotal mechanism mediating the antifibrogenic effects of adiponectin. More recently, adiponectin has been involved in the biology of hepatocellular carcinoma cells.
Chemokines in liver inflammation and fibrosis

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Chemokines represent a class of soluble immune mediators which are well known for their ability to orchestrate the recruitment of immune cells to sites of inflammation. However, in recent years a number of chemokine effector functions beyond immune cell recruitment have been identified within the liver. These include direct stimulatory effects on resident liver cells and the modulation of neoangiogenesis during liver injury.

Overall, chemokines segregate into four main families which are defined by the number of amino acids between the N-terminal cystein residues. The two largest families are the CC and the CXC chemokines. Many members of these families have been shown to be expressed during acute and chronic liver injury. Chemokines are ligands of specific G-protein coupled receptors of the same families (i.e. CC and CXC receptors), but also bind to glycosaminoglycans on the cell surface. The latter interaction seems to be necessary for immune cell infiltration into different organs, including the liver. Notably, drugs are currently developed which inhibit the attachment of chemokines to glycosaminoglycans, thereby reducing the immune cell infiltration into a damaged organ without systemic immunosuppressive effects.

Another approach to better define the role of chemokines in liver inflammation and fibrosis in vivo is the use of knockout animals for specific chemokines or their receptors. These animal models have demonstrated that the chemokine system is less redundant than previously anticipated. Using these methodologies, we have recently described an important functional role of the CC chemokine CCL5 (also known as RANTES) in chronic liver injury. In CCL5 knockout mice, acute and chronic liver injury was strongly reduced compared to wild-type animals. Interestingly, the majority of CCL5 protein was secreted by liver infiltrating T-cells, while the prime target cells of CCL5 are hepatic stellate cells. These results were in line with earlier findings that the main receptor for CCL5 is present on stellate cells and that its presence within the liver is necessary for fibrosis progression. The identification of CCL5 as a key modulator of fibrosis and inflammation within the liver suggested that its antagonization with a receptor antagonist might be able to ameliorate liver injury. Indeed, we could show that the CCL5 receptor antagonist Met-CCL5 was able to inhibit the progression of liver fibrosis and induce its regression in vivo.

CCL5 forms heterodimers with another chemokine, which is mainly stored in platelets. This chemokine is named CXCL4 and has also been shown to display profibrogenic effects in vitro and in vivo. Interestingly, we found that this chemokine is able to induce the secretion of other chemokines from activated stellate cells, thereby amplifying an inflammatory circuit within the liver. Functionally, this leads to the directed infiltration of different immune cells subtypes, including T-cells and monocytes/macrophages. As CXCL4 is only present in relevant amounts in platelets, the finding that CXCL4 mediates liver inflammation did also underline the important role of platelets in the initiation and perpetuation of liver damage and/or repair.
In summary, many lines of evidence suggest that chemokines play non-redundant functional roles in liver inflammation and fibrosis. As the first chemokine receptor antagonists have already entered the clinical arena, the translation of animal work into the first human trials is on the horizon. However, as beneficial effects of chemokines have also been identified in animal models, such trials have to be performed in well characterized populations as many chemokine effects are very context and concentration dependent.
Hepatic steatosis lubricates the slippery road from hepatic inflammation to fibrosis

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Nonalcoholic fatty liver disease (NAFLD) is recognized as the major form of chronic liver disease in adults and children. It starts with hepatocellular lipid accumulation, i.e. steatosis, and can progress with inflammation to nonalcoholic steatohepatitis (NASH). A significant number of – but not all – NASH patients develop progressive fibrosis, ultimately leading to cirrhosis, hepatocellular carcinoma and end-stage liver disease.

The Two-Hit model has been suggested to describe the development and progression of NAFLD and NASH, respectively. The first hit causes hepatic steatosis, i.e. an imbalance between hepatocellular lipid uptake on the one hand and lipid combustion and secretion on the other hand. Subsequently, a second hit is required to induce NASH, i.e. a situation where pro-inflammatory mechanisms overcome anti-inflammatory mechanisms. Here, the concept will be put forward that also hepatic fibrosis is not simply the obligatory consequence of hepatic inflammation but a "third hit" is required. Further, data and models will be presented indicating that hepatic steatosis is not benign but directly promotes fibrogenesis in NAFLD as well as other chronic liver disease.

In the majority of cases NAFLD is associated with (components of) the metabolic syndrome, characterized by central obesity and insulin resistance, and resulting diabetes type 2, dyslipidemia and hypertension. Actually, NAFLD itself is considered as a component of the metabolic syndrome, and even more than that, the fatty liver appears to be not only a passive target but in fact is cause and driving force of insulin resistance. Hereewith, NAFLD occurs in the context of a systemic disease, and thus, differs from most other liver disease. In NAFLD in addition to the liver several other organs and biological functions are affected, and these alterations directly or indirectly promote inflammation and fibrosis in fatty liver. An example is visceral adipose tissue in which expression and secretion of adipocytokine is quantitatively and qualitatively altered in NAFLD and obese patients, respectively. Expression of the adipocytokine adiponectin, which is known to be hepatoprotective and antifibrotic, respectively, is significantly reduced. In contrast, expression of the profibrogenic adipocytokine leptin is increased. Besides adipose tissue, obesity also leads to quantitative and qualitative changes of the intestinal flora and an impaired intestinal barrier, which together lead to increased translocation of bacteria and bacterial compounds, which are known to be profibrogenic. These and further examples indicate that in addition to direct hepatic effects, systemic alterations in NAFLD patients affects hepatic fibrosis.

Interestingly, also hepatocyte regeneration has been shown to be impaired in fatty livers, and steatosis promotes hepatic fibrosis in several experimental models. Of note, in addition to the amount also the type of dietary fat seems to determine these effects, i.e. there may be good and bad grease for the slippery road from hepatic inflammation to fibrosis.
Obesity or diabetes are known independent risk factors for liver fibrosis in patients with chronic disease as viral hepatitis. Certainly, one can argue that the combination of two pathological mechanisms causes more harm than either of the two alone. However, there is the question whether it is actually NASH or "simple" steatosis which promotes fibrosis progression on the ground of viral damage. The question cannot be simply answered since one can not distinguish which part or percentage of inflammation or fibrosis is caused by hepatitis viruses and which one by the metabolic syndrome. However, the epidemiology and clinical course of "pure" NAFLD make it more likely that either "simple" steatosis alone or extrahepatic pathophysiological mechanisms related to the metabolic syndrome are sufficient to promote hepatic fibrosis. In this regard it is interesting, that some studies revealed hepatic fibrosis in diabetic patients even in the absence of significant hepatic lipid accumulation. In line with this, it has been shown that insulin induces proliferation and collagen expression in hepatic stellate cells. The activation of these cells is the main cause of hepatic fibrogenesis in NAFLD as in other chronic liver disease. However, it appears that also pure fatty liver without insulin resistance has the potential to promote liver fibrosis. Thus, increased hepatic lipid accumulation results in an induction of CYP2E1, leading to an increased formation of reactive oxygen species, which may directly contribute to the activation of hepatic stellate cells. Interestingly, hepatocyte regeneration has been shown to be impaired in fatty livers, and steatosis promotes hepatic fibrosis in several experimental models. Of note, in addition to the amount also the type of dietary fat seems to determine these effects, i.e. there may be good and bad grease for the slippery road from hepatic inflammation to fibrosis.
Session II

Transplantation and regeneration
Immune regulation in the liver – From tolerance to immunity

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In the liver regulation of immune responses are achieved by various local cell populations in concert with the unique hepatic microenvironment that is characterized by the continuous presence of gut-derived bacterial degradation products that lead to local expression of immune regulatory mediators. Hepatic antigen presenting cells with immune regulatory properties comprise hepatocytes themselves as well as Kupffer cells, hepatic dendritic cells and liver sinusoidal endothelial cells (LSEC). These cells bear the capacity to regulate immune responses locally by skewing antigen-specific T cell responses. A common denominator of local hepatic immune regulation is that naïve T cells first encounter their specific antigen in the liver and not in secondary lymphatic tissue. The presentation of antigen by tolerogenic hepatic cell populations leads to different outcomes that range from generation of regulatory T cells, clonal deletion to functional incapacitation of antigen-specific CD8 T cells and local stunning of immunogenic antigen-presenting cells. Adaptive T cell immunity, however, can also be induced locally in the liver by LSEC that are virally infected. Interestingly, such change from tolerogenic to immunogenic function was not brought about by known innate immune sensing receptors such as TLRs or NLRs. Furthermore, generation of potent CTL immunity by virus-infected LSEC did not rely on conventional costimulatory signals such as IL-12 or CD80/86 but comprised novel molecules that still need to be defined. Currently, the molecular mechanisms determining induction of tolerance or T cell immunity still need to be defined in detail. This will be important, however, as deliberate modulation of the outcome of immune regulation in the liver may be used to influence or attenuate autoimmune diseases or to overcome states of incomplete local immune activation in order to eradicate persistent microbial infection or target hepatic tumors.

Literature:

NF-κB and liver regeneration

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The nuclear factor (NF)-κB is essential to preserve liver homeostasis and is a main regulator of immune and inflammatory responses. NF-κB controls the fine tuning between life and death as it has death-promoting, anti-apoptotic and pro-proliferative functions depending on the cellular source, damaging context and stimulus. NF-κB activation relies on the IKK complex (consisting of the two kinases IKK1/IKKα, IKK2/IKKβ and the regulatory subunit IKKγ/NEMO) that phosphorylates the inhibitory protein IκB, releasing NF-κB, which translocates into the nucleus where it promotes gene transcription. Deletion of NEMO in hepatocytes (Nemo\textsuperscript{Δhepa}) results in lack of NF-κB activation. Further analysis demonstrated that Nemo\textsuperscript{Δhepa} mice show chronic liver inflammation, NASH progression and HCC development and this identified NEMO as a tumor repressor molecule.

The role of NF-κB during liver regeneration remains controversial. NF-κB is immediately activated after partial hepatectomy (PH) and is thus considered as one of the immediate early genes essential to mediate transition of resting hepatocytes into the cell cycle. First in vivo results were obtained with a dominant negative I-κBα (I-κBαAA) molecule, which lacks its phosphorylation sites and thus can not be degraded and blocks NF-κB activation. After adenoviral transfer of an I-κBαAA vector these animals showed impaired liver regeneration and increased apoptosis and therefore it was thought that NF-κB is important in mediating hepatocyte proliferation. However by using the I-κBαAA construct in hepatocyte-specific transgenic mice after PH these animals showed normal liver regeneration. Therefore further experiments were important to better define the role of NF-κB activation for liver regeneration.

In our work we used 6–8 week old Nemo\textsuperscript{Δhepa} mice to better define the role of NF-κB activation during liver regeneration. We show that NEMO in hepatocytes is essential to promote liver regeneration as 50% of the animals die during the first 96 hours after PH and showed impaired and desynchronized hepatocyte proliferation. This was associated with a significant increase in oxidative stress that triggers DNA damage and hepatocyte apoptosis. As a consequence expansion of the liver stem cell compartment was evident; this rescues liver regeneration in the surviving animals. Therefore, NEMO expressing cells of the stem-cell compartment become activated after PH in Nemo\textsuperscript{Δhepa} livers and are capable to survive, proliferate and repopulate the liver in a ROS enriched environment.
Hepatic ischemia/reperfusion injury

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Adequate microcirculation including not only a sufficient nutritive perfusion and oxygen supply, but also a balanced vasomotor control and an appropriate cell-cell communication, is a prerequisite for hepatic homeostasis and intact liver function. Hepatic ischemia/reperfusion (I/R) injury occurs in a variety of clinical settings, including transplantation, liver resection surgery, trauma, and hypovolemic shock. The mechanisms of postischemic organ damage have been studied extensively during the last three decades, and consist of complex interactions of molecular, cellular, humoral, and microvascular pathways. Experimental studies have demonstrated that microcirculatory disorders are predominant determinants for hepatic dysfunction and failure upon I/R. Disorders include (i) a dysregulation of the vasomotor control with a deterioration of the endothelin-nitric oxide balance, an arterial and sinusoidal constriction and a shutdown of the microcirculation, as well as (ii) an overwhelming inflammatory response with activation of immune cells promoting inflammation and tissue damage. Within the sequelae of events, pro-inflammatory mediators, such as reactive oxygen species, cytokines and chemokines, are key players, causing microvascular dysfunction and perfusion failure. Recent research focuses on the controversy regarding which mode of cell death, i.e. apoptosis, oncotic necrosis or necrapoptosis/aponecrosis predominate in hepatic I/R injury. In addition, mechanisms by which inflammatory immune responses are initially activated through signaling molecules and their cellular receptors are currently addressed. The presentation will cover the pathophysiology of hepatic I/R injury, will provide mechanistic insights and will highlight therapeutic targets to attenuate hepatic tissue injury and organ dysfunction. It will also indicate future directions to translate the knowledge achieved from experimental studies into clinical practice.
HCC recurrence under immunosuppression after LTx

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Among the most serious complications of immunosuppressive therapy in organ transplantation is the high risk of previous neoplasia recurrence, or the development of de novo cancer. In orthotopic liver transplantation (OLT) for primary and secondary malignancy, tumor recurrence led to poor mid- and long-term results until the introduction of strict criteria for the enrollment of primary liver tumors, in particular HCC. HCC comprise 80–90% of these malignancies, and the proportion of HCC among primary hepatic malignancy recorded in the European Liver Transplant Registry (ELTR) is continuously increasing. Moreover, HCC has a high recurrence rate following OLT when the tumor exceeds 5 cm in size. Furthermore, HCC recurrence is second only to age-related cardiovascular accidents as the leading cause of late-death in liver transplant recipients. The most recent ELTR data show that HCC recurrence is the single leading cause of mortality after first liver transplantation in Europe, followed by cardiovascular accidents. Thus, HCC has become a significant cause of death in patients otherwise successfully treated by liver transplantation.

In 2003 the ELTR published 5-year patient overall survival data for hepatic malignancy of merely 53%, comparing poorly with patient survival data for non-cholestatic cirrhosis of 74% and also acute liver failure of 62%. Similar data have been published by UNOS. Implementing restrictive criteria based on tumor size and number (Milan Criteria) has produced an improvement in both overall and disease-free survival following OLT for HCC. However, published series indicate that approximately 30% of the patients believed preoperatively to be within Milan Criteria, actually proved by histopathological examination to have extended disease. This leads to a dramatic decline in overall and disease-free survival, from 71–85% to 40–50%, and from 65–78% to 27–30%, respectively. These studies also indicate that HCC recurrence occurs in the group of patients adhering to the Milan Criteria, albeit at a lower frequency.

Among the important factors that likely influence the recurrence of HCC in OLT recipients is the need for, and use of, immunosuppressive drugs to prevent organ rejection. In particular, calcineurin inhibitors have been implicated for their pro-tumorigenic properties including promotion of angiogenesis, cancer cell invasiveness and inhibition of DNA-repair mechanisms. These properties have been shown in vitro and in xenogenic tumor models in mice to promote cancer progression. Although data are relatively weak, it has also been shown that increasing doses of calcineurin inhibitors is correlated with more posttransplant cancer, and in particular, more HCC recurrence.

Recently, mammalian target of rapamycin inhibitors (mTOR-inhibitors) have demonstrated potential as both immunosuppressive and anti-cancer agents. Uniquely, mTOR-inhibitors prevent organ transplant rejection, while additionally possessing anti-cancer properties that may be useful in tipping the ‘balance of effects’ towards cancer-free survival in transplant recipients. Mechanisms of mTOR-inhibitor anti-cancer effects are complex and multiple, affecting processes including angiogenesis,
cell proliferation, cell survival, and molecular oncogenic signaling, as will be discussed in detail. Clinical studies that are currently underway are testing the hypothesis that the use of mTOR inhibitors can reduce the problem of malignancy in organ transplant recipients.

Importantly, experimental work supports the view that tumor inhibition can be accomplished with mTOR-inhibitors, while protecting allografts against rejection. Most recently, prospective-randomised clinical studies have been initiated to test the concept that mTORi reduce cancer, while simultaneously inhibiting allograft rejection. One such study, the SiLVER trial, examines hepatocellular carcinoma recurrence in mTOR-inhibitor treated liver transplant patients. More robust evidence as to whether cancer risk in transplant recipients can be reduced with mTOR-inhibitors must come from clinical trials such as this.
Session III

Autoimmune liver disease
Mechanisms regulating intrahepatic immunity

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Despite being a non-lymphoid organ, the liver displays immunological properties distinct from other solid organs and is associated with the induction of T cell tolerance. This property has been demonstrated in several clinical settings including transplantation, and hepatotropic viral infections, such as those induced by hepatitis B and C viruses (HBV and HCV).

In 2001, our group has demonstrated that the liver is an exception to the paradigm of naïve T cell trafficking: it is the only non-lymphoid organ supporting primary T cell activation independently of lymphoid tissues. These findings, now confirmed by other groups, have opened new possibilities to explain the remarkable property of the liver to induce antigen-specific tolerance in transplantation and following infection by hepatotropic viruses such as HCV and HBV.

Using transgenic mouse models, we have demonstrated that unlike primary activation in lymphoid tissues, primary activation initiated in the liver leads to deletional tolerance. Recent yet unpublished studies have revealed that 80–90% of CD8+ T cells undergoing primary activation within the liver were rapidly deleted by a non-apoptotic mechanism. Deletion resulted from T cell invasion into hepatocytes, a process leading to their rapid destruction in LAMP-1+ endosomal/lysosomal compartments. Invasion of a cell into another cell is known as “emperipolesis” (from the greek “inside round-about wandering”) and is routinely observed by pathologists in liver sections from patients with autoimmune hepatitis and viral hepatitis induced by HBV, HCV and Epstein-Barr virus infections. To distinguish it from the classical form of emperipolesis that does not imply destruction of the invading cell, we have termed this process “suicidal emperipolesis” (SE). Preliminary experiments suggest that similar findings were observed in a mouse liver transplant model suggesting that SE might play an important role in inducing tolerance in transplantation. We have recently identified new inhibitors of SE that prevent invasion of antigen-specific T cells into hepatocytes in vitro. Administration of this inhibitor in vivo resulted in the survival of antigen-specific T cells in both liver and blood and led to the development of an acute autoimmune hepatitis. Our results thus suggest that SE is the dominant mechanism by which the liver rapidly induces tolerance in the T cell repertoire. Other mechanisms, including Bim-dependent apoptosis of recently activated lymphocytes, generation of regulatory T cells, might intervene at a later stage of the response to silence cells surviving SE.
Tolerance induction in response to liver inflammation

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The liver is a privileged organ regarding immune regulation and tolerance induction, since it is permanently exposed to gut-derived microbial or dietary antigens. Its ‘scavenger’ function circumvents any dispensable and inadequate immune activation to prevent liver damage. Intestinal antigens are not ignored by the immune system; rather the liver has been considered to favour the induction of peripheral tolerance. However, immune responses induced to eliminate hepatotropic pathogens such as hepatitis viruses or to clear foreign antigens and toxins may result in organ damage. Here, we investigated mechanisms of immunoregulation and tolerance induction in response to liver inflammation in a murine model of immune-mediated liver injury inducible by injection of the plant lectin concanavalin A (ConA). ConA hepatitis is mediated by activated CD4\(^+\) T cells, NKT cells, and Kupffer cells releasing IFN\(\gamma\) and TNF\(\alpha\). Tolerance develops towards ConA rechallenge within 8 to 10 days, lasting for several weeks and is characterized by significantly reduced plasma transaminase activities, decreased Th1/Th17 responses and an increased IL-10 release. In the tolerogenic state IL-10 is produced by CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T cells (Tregs) and Kupffer cells. Moreover, Tregs from ConA-tolerant mice display a higher immunosuppressive potential in vitro and in vivo compared to those from non-tolerant animals. Co-culture experiments with hepatocytes from ConA-tolerant mice and activated CD4\(^+\) responder T cells from saline treated mice revealed that hepatocytes from tolerant mice completely suppressed IL-2 release and significantly induced expression of Foxp3 and production of IL-10 to a higher degree than control hepatocytes. This feature of hepatocytes from tolerant mice depended on endogenous IFN\(\gamma\) production. Moreover, mice which lack Th1 specific chemokine receptors, i.e. CCR5\(^-/-\) or CXCR3\(^-/-\) mice, were more susceptible towards ConA hepatitis and failed to develop ConA tolerance. In CXCR3\(^-/-\) mice, the lack of tolerance induction depended on the inability to convert Tregs to Foxp3\(^+\)Tbet\(^+\)IL-10\(^+\) Tregs in vivo. Accordingly, Tregs of these mice did not accumulate within the liver and failed to prevent ConA hepatitis upon adoptive transfer although they retained their immunosuppressive features in vitro.

In conclusion, ConA tolerance is mediated by induced IL10\(^+\) Tregs probably trafficking into the liver depending on the IFN\(\gamma\)-inducible chemokine receptors CCR5 and CXCR3. Hence, induced Tregs are probably tissue specific depending on the type of immune response, local antigen-presenting cells as well as on the local cytokine and chemokine milieu.
Autoimmune hepatitis (AIH) is a severe autoimmune inflammation that needs lifelong immunosuppression. The standard therapy has strong side-effects such as an increased risk for Cushing’s syndrome and osteoporosis. In addition 20% of the patients are non-responders to the therapy. Due to the fact that AIH is often recognized during late course of disease, it is difficult to obtain knowledge about the immunological mechanisms responsible for initiation of the disease. Current AIH models were helpful for understanding and modulating liver immune responses but are not suited to study mechanisms in chronic AIH or to develop new therapies. While transgenic AIH models deal with short-term hepatitis, models with natural antigens are either self limited or have unknown target antigens. We therefore tested the role of a strong heterologous stimulus. The similarity but not identity of the foreign antigen is important, brought in during liver specific infection in a genetically susceptible host. To this end we generated replication-deficient adenoviral constructs expressing common autoantigens of human AIH showing homology up to 80% with the murine proteins. Following an acute phase of hepatitis with increasing levels of transaminases for a period of two weeks after intravenous injection of the virus, we could show chronic evolving hepatic autoimmune reactions after twelve weeks within the liver of non-obese diabetic (NOD) mice which received viral constructs expressing formiminotransferase cyclodeaminase (FTCD), an important auto-antigen in AIH type II. Surprisingly the more important AIH type II auto-antigen CYP2D6 as well as the type III soluble liver antigen (SLA) did not result in any chronic hepatic immune reaction. This strain and antigen-specificity of murine AIH supports the notion that autoimmunity develops in genetically predisposed individuals. Control experiments with expression vectors devoid of viral components did not result in any hepatic reaction after the acute viral infection had been cleared. Chronic liver disease was confirmed by consistent leukocyte infiltrates and antigen-specific autoantibodies within the sera. Immunofluorescent staining of liver sections revealed that CD4+ T cells are more abundant than CD8+ T cells in these liver infiltrates. To identify the leukocyte populations involved in hepatitis within our model we isolated liver-specific leukocytes of hepatitis-bearing mice and analysed these by flow cytometry. This analysis detected no differences in the composition of liver-specific leukocyte populations between AIH-bearing mice and control infected animals. However ELISPOT assays revealed more antigen-specific CD4+ T cells in Ad-FTCD treated mice than in control mice. Furthermore the disease was adoptively transferrable by CD4+ T cells into immune-deficient hosts confirming their role in chronic AIH. By assessing mice treated with Ad-FTCD for more than 30 weeks we observed that the induced chronic autoimmune hepatitis results in hepatocellular fibrosis. This effect is comparable to the human situation in untreated and non-responsive patients.

In our new chronic autoimmune hepatitis (CAH) model we were able to break humoral and T cell tolerance against liver self-proteins leading to chronic autoimmune hepatitis and even to fibrosis. The model shows the importance of the
genetic background in predisposed hosts. It is demonstrating the impact of a viral infection for the induction phase of hepatitis and, moreover, that antigens similar to self-antigens can break liver tolerance. In addition this novel model might open new therapeutic options to treat the disease with less side effects or improved responsiveness.
Therapeutic strategies for autoimmune hepatitis

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Introduction

Autoimmune hepatitis (AIH) represents a chronic, mainly periportal hepatitis upon histology, which is characterized by a female predominance, hypergamma-globulinemia, circulating autoantibodies and a benefit from immunosuppressive treatment [1, 2]. In about 25% of patients with AIH, an acute onset is observed, while only few cases of fulminant AIH have been reported. However, autoimmune hepatitis must be considered in the differential diagnosis of acute liver failure. Most patients suffering from AIH show variable, unspecific clinical symptoms like fatigue, lethargy, jaundice, right upper quadrant pain which do not differ from those of other forms of hepatitis. With progression of disease, symptoms of portal hypertension, i.e. ascites, esophageal varices, hypersplenism, and encephalopathy may dominate. AIH can be associated with a variety of extrahepatic immune-mediated symptoms and diseases which affect about 25% of patients. Among the most frequently observed extrahepatic associations of AIH are autoimmune thyroiditis, arthritis, ulcerative colitis, sicca syndrome and synovitis. The natural history and prognosis of AIH are largely defined by inflammatory activity present at the onset of disease and by the presence or development of cirrhosis [3]. When a 5–10 fold elevation of aspartate aminotransferase and 2-fold increase of gamma-globulins exist, the mortality without treatment is an estimated 90% in 10 years. In patients with periportal hepatitis, cirrhosis develops in 17% within 5 years while patients with bridging necrosis or necrosis of multiple lobules develop cirrhosis in 82% within 5 years. The presence of cirrhosis is associated with a mortality of 58% in 5 years. The HLA antigen profile also significantly contributes to the clinical outcome of AIH. The presence of HLA DR3 is associated with a more severe course of disease. In contrast, HLA DR4 is associated with a later age at onset and a more benign outcome of AIH. The revised AIH diagnostic score (table 1) contributes to the establishment of the diagnosis in difficult cases by calculating a probability expressed as a numeric score [1]. A more simplified scoring system has been published recently [4].

Treatment Indications for Autoimmune Hepatitis

An absolute treatment indication is present when aminotransferase levels exceed ten times the upper normal value or when both elevated aminotransferase levels (≥ 5-fold of upper normal limit) and gamma-globulin levels (≥ twice of upper normal value) exist. Histological evidence of bridging necrosis or multilobular necrosis as well as severe hepatic and extra hepatic symptoms represent also absolute treatment indications. [3, 14].

Induction of Remission

The standard initial treatment of AIH is either prednisone monotherapy (50 mg/day and tapering regimen) or combination therapy with prednisone (30 mg/day) and azathioprine [6]. While in the US a flat dose of 50 mg was used for azathioprine a higher dose of 1–2 mg/kg bodyweight is often used in Europe. Both are equally effective in the induction of remission, although combination therapy is generally preferred because it allows for the reduction of the prednisone dose to frequently
below 10 mg and thereby reduces the steroid-associated unwanted side effects. Thus, combination therapy is preferred for elderly, osteoporotic patients, patients with a metabolic syndrome, and with psychiatric lability. Conversely, since azathioprine mainly causes hematological side effects such as leukopenia or less frequently anemia, monotherapy with steroids would be preferred in patients with hematological abnormalities or proven homozygous deficiency of thiopurine methyltransferase. Steroids are also preferred for short treatment trials in patients with relative treatment indications or when response to immunosuppressive treatment is used as diagnostic criteria. Furthermore, since teratogenicity and oncogenicity have been demonstrated for azathioprine in animal models, steroid monotherapy is recommended for patients with active malignancies and during pregnancy or contemplation of pregnancy. While steroid monotherapy is equally effective as the combination therapy with azathioprine to induce remission, azathioprine monotherapy is ineffective for inducing remission and should not be attempted. Remission, nowadays, is defined as a complete biochemical and histological resolution of inflammation as well as the disappearance of clinical symptoms [3]. In the US reduction of aminotransferases to less than 2 times the upper limit of normal (ULN) has been applied as treatment goal for the last decades. However, normal transaminases are now the goal of therapy according to the latest 2010 AASLD practice guidelines on autoimmune hepatitis [3]. During therapy, aminotransferase levels should normalize within 3–6 months. Histological remission usually lags 3–6 months behind normalization of biochemical markers. Therefore, biopsy-proven remission is the ultimate goal of all treatment regimens. Recently, the topical steroid budesonide was shown to induce and maintain remission in combination with azathioprine with less steroid specific side effects in non-cirrhotic patients with AIH [7]. The use of budesonide as an alternative steroid is discussed below.

**Maintenance of Remission**

If histological remission is documented, the risk of relapse is only 20%. However, in biopsies that still show portal hepatitis, relapse occurs in 50% within 6 months after the end of treatment. Overall, the frequency of relapse is 50% in 6 months and 70% in 3 years after withdrawal of immunosuppressive therapy. A sustained response is achieved in only 17% of patients. A previous study demonstrated that patients who only received steroid therapy at maintenance doses of 5 to 12,5 mg/day showed a significantly higher probability of relapse within 3 years than patients who continued on combination therapy or azathioprine treatment alone [8]. In a further study that included patients who were treated with azathioprine (2 mg/kg/day) alone after withdrawal of prednisone, biochemical and histological remission was sustained in 83% during a 10-year follow up [5]. In summary, azathioprine maintenance after prednisone withdrawal reduces the likelihood of relapse. Maintenance therapy of patients who have achieved complete remission should be performed for at least 2 or better 3 years and histological remission should be documented before withdrawal of immunosuppressive therapy. Prednisone withdrawal should proceed gradually over a period of 3–6 months. In patients showing progressive fibrosis the consequences of a relapse should be considered and the benefit-risk ratio may be too low to justify treatment termination in these patients. The combination of the topical steroid budesonide plus azathioprine allows maintenance of remission with minimal steroid specific side effects in non-cirrhotic AIH patients [7].

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Alternative Treatment Strategies

Treatment failure is characterized by the deterioration of biochemical and clinical signs during therapy. In about 10–15% of patients the standard therapy failed although well tolerated. If standard treatment fails an alternative therapy with one of the following drugs can be considered.

Cyclosporin A (CyA): CyA has been reported to be effective in a small number of adult AIH patients who failed to respond to standard treatment. However, in these studies relapses have occurred upon drug withdrawal. Another study showed a significant improvement of histological and biochemical disease activity in 19 patients (9 treatment naïve) treated with CyA over 6 months [10]. In a multi-centre study, treatment of 32 children with type 1 AIH and of 4 children with type 2 AIH with CyA administered at doses of 2–3 mg/kgBW/day over 6 months, which was followed by application of low doses of prednisone and azathioprine for 1 month after CyA withdrawal, resulted in histological improvement [11]. In summary, CyA appears to be well tolerated in both type 1 and type 2 AIH. However, its toxicity profile that occurs particularly with long-time treatment limits the widespread use of this drug. However, this observation is not specific for CyA, but appears to be a common phenomenon of immunosuppressive agents.

Tacrolimus (FK 506): This agent is a potent macrolide lactone compound with an immunosuppressive activity exceeding that of CyA. The mechanism of action is similar to those of CyA, however, FK506 binds to a different immunophilin (FK binding protein) leading to the inhibition of the synthesis of lymphokines (IL-2, IL-3 and IFN-gamma), of the IL-2 receptor expression as well as of the generation of cytotoxic T-cells. Although larger randomized trials are required, FK506 represents a promising immunosuppressive agent. The side effects resembles those of cyclosporine A.

Budesonide: This synthetic steroid is characterized by a high first pass metabolism in the liver and therefore has reduced systemic side effects compared to conventional steroids. In one study treating 13 AIH patients with 6–8 mg of budesonide per day, tapering to 2–6 mg/day after 6 to 10 weeks for a whole treatment period of 9 months, biochemical improvement (ALT and serum IgG) without steroid side effects was demonstrated [12]. However, budesonide did not offer an advantage over conventional steroids when cirrhosis and portosystemic shunts are present [13]. In another study [14] 10 AIH patients that were steroid dependent received budesonide (9 mg/day) to prevent disease exacerbation. However, only 3 of the treated patients entered clinical and biochemical remission whereas the other 7 patients either deteriorated during therapy or became drug intolerant. However, it cannot be expected that budesonide treatment will overcome treatment failure following conventional combination therapy with prednisone and azathioprine. The main advantage of a topical steroid like budesonide might be the replacement of conventional steroids in long-term maintenance therapy to reduce systemic steroid specific side effects [7, 15]. However, such a benefit should only be observed in non-cirrhotic patients without any portosystemic shunting.

Mycophenolate Mofetil: This compound is an ester prodrug of mycophenolic acid which is a noncompetitive and reversible inhibitor of inosine monophosphate dehydrogenase. This enzyme catalyses the conversion of inosine monophosphate to
xanthosine monophosphate and therefore leads to a depletion of guanine nucleotides and ultimately to the inhibition of DNA synthesis. In contrast to other cell types, de novo synthesis of purines is essential for B- and T-cell proliferation which is the reason why mycophenolate mofetil exerts its cytotoxicity mainly on these cell populations. Side effects are predominantly gastrointestinal (nausea and diarrhoea) and hematological (leukopenia, thrombocytopenia), however the risk of developing CMV infections is 13.5% and therefore also markedly increased. In one study [16], patients with AIH type 1 refractory to standard therapy received mycophenolate mofetil (2 g/day) in addition to prednisolone over a median follow-up of 46 months. Five patients showed normal aminotransferases after 3 months of treatment. In a recently performed multi-centre study, 11 patients non-responding or intolerant to standard therapy were treated with mycophenolate mofetil together with prednisolone. Complete biochemical remission was achieved in 63% of patients and the prednisolone dose could be reduced to frequently below 10 mg per day [17]. These preliminary data suggest that mycophenolate mofetil alone, or together with prednisolone may represent an alternative treatment strategy in the induction and maintenance of remission especially for those patients intolerant azathioprine therapy. A recent retrospective European study confirms this observation and underlines that mycophenolate mofetil is helpful to overcome azathioprine intolerance rather than resistence [18]. However, larger randomized prospective studies are required to evaluate the efficacy of mycophenolate mofetil in induction and maintenance of remission compared to the standard therapy with azathioprine.

References:


Session IV

Viral hepatitis
Viral hepatitis: Success and failure of virus-specific CD8\(^+\) T cell responses

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CD8\(^+\) T cells are thought to be the primary effector cells of the antiviral immune response in hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. Indeed, depletion studies in both infections have shown that CD8\(^+\) T cells are required for successful viral elimination. In addition, depletion studies have also shown that CD4\(^+\) T cell help is required for viral elimination since viral persistence evolves in the absence of CD4\(^+\) cells. CD8\(^+\) T cells mediate their antiviral effects by cytolytic (e.g. lysis of infected cells) and non-cytolytic (e.g. IFN\(\gamma\)-mediated inhibition of viral replication) effector functions.

How the viruses manage to avoid these immune responses and establish life-long persistence is still a mystery. It appears that several different mechanisms contribute to virus-specific CD8\(^+\) T cell failure: First, the emergence of viral escape mutations can lead to inhibition of antigen recognition by antiviral T cells. Second, the co-expression of inhibitory receptors (e.g., PD-1, 2B4, KLRG1) significantly contributes to CD8\(^+\) T cell exhaustion and dysfunction. Importantly, pharmacologic blockade of these inhibitory signaling pathways results in an augmentation of T cell function, thus, indicating that these pathways are a promising target for novel antiviral therapies. Third, the tolerogenic properties of the liver may induce impaired T cell priming. In addition, depletion of amino acids (e.g., arginine) in the liver due to enzyme release from dying hepatocytes may impair T cell activation. Fourth, immunosuppressive cytokines such as IL-10 and regulatory T cells contribute to the inhibition of antiviral CD8\(^+\) T cell effector functions. Finally, a lack of CD4\(^+\) T cell help seems to be a hallmark of virus-specific CD8\(^+\) T cell failure in HBV and HCV infection.

In sum, although there is growing consensus that virus-specific CD8\(^+\) T cells are essential for viral clearance and disease pathogenesis in HBV and HCV infection, several central questions of HBV and HCV immunobiology still need to be addressed, e.g. the relative contribution of the different mechanisms to the success and failure of virus-specific CD8\(^+\) T cell responses in HBV and HCV infection.
Immune control of hepatitis B virus

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Human hepatitis B virus (HBV) infects the liver of humans or homonoid primates. In humans, HBV infection often causes an inflammatory liver disease – hepatitis B. The virus is transmitted by perinatal, percutaneous, and sexual exposure, as well as by close person-to-person contact. The latter occurs especially among young children presumably by open cuts or sores. Vertical transmission from mothers to their neonates, or infection during the first year of life, results in persistent, often life-long infection in > 90%. In contrast, infection during adulthood is cleared in > 90% of cases, and results in life-long protective immunity.

While a correlation between the strength of HBV-specific CD4 and CD8 T cell responses and virus clearance has been established, factors determining the strength of a T cell response and factors shifting the balance from immune tolerance to immune clearance are hardly understood. The innate immune response, early adaptive B- and T-cell responses, regulatory T cells, the liver microenvironment as well as peculiar properties of hepatocytes and non-parenchymal liver cells to present antigen seem to play a role.

Understanding this complex interplay requires systematic immune monitoring of well characterized human cohorts, but also experimental approaches using primary human cells and genetically modified mouse models. Using these models we begin to understand immune recognition of HBV and how it influences the outcome of HBV infection.
Innate immune responses to hepatitis C virus

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The host immune response plays a unique role in HCV infection because of its potential to contribute not only to viral clearance but also to liver injury. HCV balances this equation by attenuating both innate and adaptive immune responses, thereby reducing the likelihood of viral clearance as well as the degree of immune-mediated liver injury and allowing co-existence of both virus and hosts. This process is continually optimized in each individual host, as evidenced by the elaborate mechanisms that HCV has developed to escape from immune responses.

Innate immune responses are mediated by the infected hepatocytes themselves, by their uninfected neighbors and by innate immune cells such as Kupffer cells, dendritic cells, natural killer cells and natural killer T cells that are enriched in the liver, and make up most of the early antiviral defense. Genetic factors such as the IFN lambda (IL28B) and killer immunoglobulin receptor (KIR)/human leucocyte antigen (HLA) genetic variants that were identified in recent genome-wide association studies contribute to the innate responsiveness at both the individual and population level. This presentation will summarize innate immune responses and viral escape mechanisms that are relevant for spontaneous hepatitis C virus clearance and for the response to therapy.
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POSTER ABSTRACTS

Poster Numbers 1 – 26

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Adenovirus-Nov gene transfer does inhibit hepatocyte EMT in vitro but fails to attenuate liver fibrosis in experimental BDL model

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Introduction: NOV/CCN3 a matricellular protein of the CCN family, comprises six secreted proteins associating specifically with the extracellular matrix. CCN proteins lack specific high-affinity receptors but signal through integrins and proteoglycans and regulate crucial biological processes including fibrosis. Contrary to CTGF/CCN2, the biological role of NOV/CCN3 in liver fibrosis remains opaque.

Methods: We generated adenovirus expressed Nov (Ad5-CMV-Nov) for gene transfer in primary hepatocytes, hepatic stellate cells (HSC) and conducted a bile duct ligated fibrotic liver experiment.

Results: Primary hepatocytes overexpressed NOV/CCN3 leading to diminished levels of CTGF/CCN2, a profibrotic protein and prevents TGF-β to induce EMT in cultured hepatocytes. By contrast, in culture-activated HSC Ad5-CMV-Nov failed to reduce CTGF/CCN2, collagen type I and α-SMA production. Ad5-CMV-Nov gene transfer in BDL mice showed high NOV/CCN3 in both mRNA and protein levels, but did not attenuate liver fibrosis.

Discussion/Conclusion: New evidence demonstrates NOV/CCN3 overexpression in mesangial cells of kidney to markedly decrease CTGF/CCN2 and block ECM accumulation, but failed to confirm in HSC. During transdifferentiation HSC produce significant amounts of NOV/CCN3, possible reason why we found no difference from controlled cultures, whereas primary hepatocytes normally show no endogenous NOV/CCN3 expression. Overexpression of NOV/CCN3 exerted its full Yin-Yang effects to its CTGF/CCN2 counter part, but in vivo NOV/CCN3 showed no antifibrotic effects, in line with the observation that overexpressed CTGF did not induce spontaneous fibrosis in liver. Treatment for liver fibrosis therefore should be found in combined regimen considering all specific cytokines and growth factors involved.
Monitoring liver function tests (LFTs) in inflammatory bowel disease (IBD) patients: Are we doing this appropriately?

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Introduction: Diseases of the liver and biliary tract are common extra-intestinal manifestations of inflammatory bowel disease (IBD). Primary sclerosing cholangitis (PSC) is one of the more common hepatobiliary complications of IBD. The diagnosis of PSC is important because of the greater risk in the development of colorectal cancer and cholangiocarcinoma therefore requiring screening for these in clinical practice. Drug-induced hepatotoxicity caused by IBD treatments such as azathioprine, infliximab and 5-ASAs is also well documented. Abnormal LFTs maybe present in up to 30 percent of patients with IBD and should be investigated for in clinic.

Methods: The aim was to assess whether IBD patients in our clinics were having their LFTs monitored at least on an annual basis. This was a retrospective assessment of IBD patients attending out-patients clinic in a district general hospital. Data was obtained using the hospital Powerchart system and our database of IBD patients. Each patient was checked on the system and scrutinized to see if LFTs were performed within the previous 12 months and when abnormal (i.e. raised ALT/ALP) whether a liver screen and ultrasound liver was completed.

Results: A total of 283 patients with IBD were identified of which 211 (75%) had ulcerative colitis (UC) and 72 (25%) had Crohn’s disease. Of the 211 patients with UC, 138 (65%) had their LFTs checked, 73 (35%) having no record of LFTs checked. Of the 138 patients, 4 had abnormal LFTs with the remaining 134 (97%) having normal tests. Of these 4 patients, 1 had a diagnosis of gallstone disease by USS but no liver screen and 1 had a normal USS but only a ferritin and hepatitis screen done as part of the liver database. 2 patients had no screen/USS and had no formal diagnosis to explain their abnormal tests. Of the 72 Crohn’s patients, 56 (78%) had their LFTs checked, 16 (22%) having no record of LFTs checked. Of these, 53 (95%) had normal LFTs. Of the 3 patients with abnormal LFTs, 2 did not have a formal diagnosis by use of the Liver screen and USS, with the remaining patient having metastatic colon cancer on USS.

Discussion/Conclusion: Liver function tests are poorly monitored in IBD patients with 35% of UC patients and 22% of Crohn’s patients attending clinic follow-up not having their LFTs checked. Even when LFTs are checked a formal diagnosis is not investigated. Important idiosyncratic and extra-intestinal manifestations of IBD with clinically relevant management for screening for colorectal cancer and cholangiocarcinoma may be missed by not screening for abnormal LFTs. We propose that all IBD patients attending clinic should have their LFTs checked at least annually.
TGF-β mediates epithelial-mesenchymal transition of hepatic progenitor cells thus contributing to HBV-associated liver fibrogenesis

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Introduction: In HCV- and NASH-associated liver diseases, hepatic progenitor cells (HPC) promote a periportal ductular reaction (DR) and contribute to periportal fibrogenesis. The mechanisms that mediate HPC activation/DR participating to fibrogenesis are presently unknown. We hypothesized that HPCs/DR participates in fibrogenesis via TGF-β-induced epithelial-mesenchymal-transtion (EMT) manner in HBV patients.

Methods: To quantify HPCs and DRs, we analysed cytokeratin-19 (CK19) and cytokeratin-7 (CK7) expression in 110 chronic HBV-infected liver tissues specimens.

Results: CK-7 staining significantly correlated with inflammatory grades (r = 0.47, P < 0.001), fibrotic stages (r = 0.53, P < 0.001) and phospho-Smad2 staining, the latter indicating active TGF-β signaling (r = 0.24, P < 0.01). Nine-month IFN-γ treatment, which attenuated TGF-β signaling, decreased numbers of HPC in 13/18 patients with chronic HBV infection. Confocal microscopy analysis revealed co-localisation of "activated" phospho-Smad2/3 with CK7/CK19, suggesting activated TGF-β signaling in HPCs. In addition, positive staining for S100A4, a marker of EMT, was found in CK7/CK19 positive HPCs of HBV patients. In line with in vivo findings, TGF-β treatment induced a number of EMT markers in cultured HPC cell line, e.g. decreased E-cadherin, increased twist. The number of oval cells (HPCs in rodents) was increased in 2-week of CCl₄ challenged mice, which lack a functional Smad7 negative feedback regulation, whereas decreased in 8-week of CCl₄ administrated Smad7 transgenic mice compared to controls, further supporting a critical role for TGF-β in HPC activation and DR.

Discussion/Conclusion: We hereby describe a mechanism for HPC activation/DR contributing to liver fibrogenesis involving TGF-β signaling and EMT in HBV infected patients.
Hepatic differentiation of adipose-derived mesenchymal stem cells reduces recruitment of immune cells after transplantation into livers of CCl₄ treated mice

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Introduction: Transplantation of hepatocytes is a therapeutic approach for diverse acute and chronic liver diseases. The limited availability of primary cells raises the need for alternatives, e.g. hepatocyte-like cells obtained by stem cell technology. Among which adipose-derived mesenchymal stem cells (Ad-MSCs) represent a promising source, as they are easily assessable and can be obtained in sufficient amounts.

Methods: Human Ad-MSCs were isolated according to ethical guidelines of the MRI. Hepatocyte-like cells (Ad-HLCs) were generated by supplementing the medium with 5-azacytidine, FGF-4, dexamethasone, nicotinamide, ITS, HGF and EGF for 18 days. Urea and glucose metabolism was measured with colorimetric and phase I and II enzyme activities with fluorescent based assays. After labeling with red fluorescent Dil Ad-HLCs and Ad-MSCs (0.5 x 10⁶ cells/mouse) were injected into CCl₄ treated C57/Bl6 or Scid/beige mice via the spleen. After 4, 10 and 21 days mice were sacrificed and livers were analyzed for the presence of the transplanted cells by 3D-confocal microscopy.

Results: Ad-HLCs were able to metabolize ammonium-chloride to urea and perform glucose metabolism comparable to primary human hepatocytes. Phase I and II enzyme activities reached levels up to 80% of human hepatocytes. 4 days after transplantation into CCl₄ treated mice, cells were found mainly within hepatic sinusoids. From day 10 on a minor fraction, particularly of the Ad-HLCs, also integrated into parenchymal tissue. Interestingly, transplanting undifferentiated Ad-MSCs led to a massive accumulation of immune cells within the livers of C57/Bl6 mice. In case of Ad-HLCs, significantly less immune cells were recruited.

Discussion/Conclusion: Our data show that hepatic differentiation seems to protect Ad-MSCs to some extent from the mice’s immune reaction. Further investigation is necessary to investigate whether loss of surface markers on the differentiated Ad-MSCs allows the immune escape.
Cytokine-mediated liver inflammation and viral load modulated by statin treatment in patients with chronic hepatitis C

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Introduction: Cytokine-mediated cellular immune response plays a key role in viral hepatitis C (VHC) immunopathogenesis and that statins can inhibit viral replication.

Aim: To assess the ability of statins to inhibit the replication of HCV RNA in patients with chronic VHC and to establish their effect upon the cellular immune response components (cytokines IL-6, IL-8, IL-10, TNF-α).

Methods: Thirty patients with VHC and detectable viral load were treated for 4 weeks with fluvastatin, 40 mg/day (13 patients – group F) or lovastatin 20 mg/day (17 patients – group L). HCV RNA levels, liver tests, cholesterol, triglycerides, IL-6, IL-8, IL-10 and TNF-α were measured at baseline and at 4 weeks.

Results: The viral load decreased in 61.53% of the patients from group F and in 64.70% of patients from group L. Serum levels of IL-6, IL-10 were maintained in normal limits in all patients. IL-8 levels normalized in 91.66% patients from group F and in 76.47% in group L. Also, 72.72% of the patients with normal IL-8 levels from group F registered a decrease of the viral load compared to 69.23% of the patients from group L. TNF-α plasma levels decreased in both groups in 53.84% and 52.94% respectively.

Discussion/Conclusion: Statin treatment can inhibit the replication of the HCV. Thanks to the data that we obtained concerning TNF-α and the aforementioned interleukins we can say that statins also appear to have an anti-inflammatory action and that targeting this component of the cellular immune response might be beneficial for limiting the pathologic process of VHC.
Oncostatin M produced in Kupffer cells in response to PGE₂: Possible contributor to hepatic insulin resistance and steatosis

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Introduction: Hepatic insulin resistance is a major contributor to hyperglycemia in metabolic syndrome and type II diabetes. It is caused in part by the low-grade inflammation that accompanies both diseases, leading to elevated local and circulating levels of cytokines and cyclooxygenase products such as prostaglandin E₂ (PGE₂).

Methods: Rat Kupffer cells were stimulated with PGE₂ for 6 h. Primary rat hepatocytes were preincubated with PGE₂ or OSM and subsequently stimulated with insulin. Insulin signaling and gene expression were determined by western blot and quantitative PCR, respectively.

Results: In a recent study, PGE₂, which is produced in Kupffer cells, attenuated insulin-dependent glucose utilization by interrupting the intracellular signal chain downstream of the insulin receptor in rat hepatocytes. In addition to directly affecting insulin signaling in hepatocytes, PGE₂ in the liver might affect insulin resistance by modulating cytokine production in non-parenchymal cells. In accordance with this hypothesis, PGE₂ stimulated in the current study oncostatin M (OSM) production by Kupffer cells. OSM in turn attenuated insulin-dependent Akt-activation and, as a downstream target, Glucokinase induction in hepatocytes, most likely by inducing SOCS3. In addition, OSM inhibited synergistically with PGE₂ the expression of key enzymes of hepatic β oxidation and VLDL-assembly. COX2 and OSM mRNA were induced early in the course of the development of non-alcoholic steatohepatitis (NASH) in mice.

Discussion/Conclusion: Thus, induction of OSM production in Kupffer cells by an autocrine PGE₂-dependent feed-forward loop may be an additional, thus far unrecognized, mechanism contributing to hepatic insulin resistance and the development of NASH.
Immunological cluster analysis in Abcb4 knockout mice as a model of progressive intrahepatic cholestasis: Peripheral leukocyte populations discriminate the mutant phenotype

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Background: Patients with functional ABCB4 variants display heterogeneity in disease progression and variable intrahepatic immune response. Abcb4 knockout mice (Abcb4−/−) represent a well-established model for the analysis of systemic effects of progressive familiar intrahepatic cholestasis (PFIC) type 3. Our aim was to systematically survey the immune response to progressive liver injury in the Abcb4−/− mouse model and to define the differences in peripheral leukocyte subpopulations.

Method: Twenty FVB-Abcb4−/− and BALB-Abcb4−/− mice aged 16–20 weeks as well as 20 control animals of each background were phenotyped in comprehensive clinical and immunological screens (Nat Methods. 2005; 2: 403). Leukocyte subpopulations of peripheral blood were determined by FACS analysis, and data were subjected to cluster analysis.

Results: Both Abcb4−/− strains develop chronic cholangitis and biliary fibrosis, and white blood cell counts were significantly increased as compared to wild-type controls. FACS analyses revealed characteristic changes in leukocyte populations dependent on background and genotype. Knockout mice showed enhanced CD4+ T-lymphocyte counts, whereas the relative proportions of granulocytes (BALB/cJ) and CD19+ B-cells (FVB/NJ) were decreased. Knockout mice on the FBV/NJ background showed more pronounced changes in both hepatic and haematological phenotypes. Of note, cluster analysis of relative frequencies of peripheral leukocyte subpopulations stringently discriminated mutant and wild-type mice.

Conclusions: Abcb4−/− mice display a specific immune response to chronic cholangitis. The characteristic alterations of leukocyte subpopulations in serum correlate with liver injury and depend on genetic background. Cluster analysis of peripheral leukocyte populations might serve as novel surrogate maker for the assessment of PFIC susceptibility and progression.
Clinical significance of hyponatremia in patients with liver cirrhosis

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Introduction: Low serum sodium concentration is an important predictor of mortality in patients with cirrhosis awaiting liver transplantation and one of diagnostic criteria of hepatorenal syndrome. The aim of our study was to assess the occurrence of hyponatremia, its predisposing factors and association with the severity and complications of liver cirrhosis.

Methods: We studied 162 patients (pts) – 115 (71%) men and 47 (29%) women, admitted because of complications of liver cirrhosis. Pts’ mean age was 54.9 ± 12.7 years. They were divided into three groups according to their serum sodium values: 1) ≥ 136 mmol/L; 2) 131–135 mmol/L; 3) ≤ 130 mmol/L. We have performed evaluation of the grade of ascites and its susceptibility to diuretics, impairment of renal function (creatinine normal values ≤ 1.3 mg/dL), severity of liver cirrhosis according to Child-Pugh and MELD scores, the cause and symptoms of hyponatremia.

Results: Hyponatremia was found in 72 of 162 (44.4%) pts, in 53 of 115 (46.1%) men and 19 of 47 (40.4%) women. Forty-five of 162 (27.8%) pts were included to the 2nd group and 27 of 162 (16.7%) pts to the 3rd group. Ascites defined as grade I, II or III (classification of the International Ascites Club) was present in all pts with hyponatremic pts: in 3 of 45 (6.67%) pts of the 2nd group and in 7 of 27 (25.92%) pts from the 3rd group (p = 0.028). Classifying pts according to Child-Pugh score we found: in the 1st group – 9 of 90 (10.0%) pts of Child A class, 48 of 90 (53.3%) of Child B class and 33 of 90 (36.7%) of Child C class, in the 2nd group – 2 of 45 (4.4%) pts of Child A class, 14 of 45 (31.1%) Child B class, 29 of 45 (64.4%) Child C class, and in the 3rd group – 0 of 27 (0%) pts of Child A class, 10 of 27 (37.0%) of Child B class and 17 of 27 (63.0%) of Child C class. The highest mean Child-Pugh and MELD scores were observed in the third group (8.8 ± 1.58 vs 9.5 ± 1.7 vs 10.1 ± 1.8; respectively, p = 0.001) and (14.1 ± 4.3 vs 16.6 ± 5.6 vs 18.92 ± 4.89, respectively; p < 0.0001). None of pts with hyponatremia had clinical, neurological signs indicating cerebral oedema.

Discussion/Conclusion:
1. The serum sodium level was significantly associated with the severity of liver disease as assessed by Child-Pugh and MELD scores.
2. Deep hyponatremia (≤ 130 mmol/L) may indicate greater frequency of refractory ascites and impaired renal function.
3. There was no significant difference in the prevalence of hyponatremia between men and women.
4. Hyponatremia was caused mostly by diuretics, rarely by excessive water supply.
5. Chronic low serum sodium level was usually asymptomatic.
Frequency of CD3+CD4+IL17+/Th17 cells in patients with alcoholic liver disease.

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Introduction: There is evidence that immune responses and liver T cells infiltrates occur in the course of alcoholic liver disease (ALD). IL-17 producing CD4+ T (Th17) cells have been identified to play a crucial role in a few human inflammatory diseases. Their significance in pathogenesis of ALD remains unclear. The aim of our study was to assess the possible involvement of Th17 cells in ALD.

Methods: We studied the frequency of CD3+CD4+IL17+/Th17 cells in peripheral blood of 45 patients with ALD in comparison with 16 healthy controls (HC). The study population consisted of 14 women and 31 men, mean age 50.89 ± 11.37 years (range 28–69 years). Pts were divided according to their Child-Pugh score into 3 groups: A: 7 pts; B: 22 pts; C: 16 pts. Flow cytometric analysis FACS Calibur with CellQuest software was used to identify T cell phenotype. CD3+CD4+IL17+ cells were considered Th17 cells and expressed as the percentage of all CD3+CD4+ cells. All data were analysed using Statistica 8.0 software.

Results: The mean MELD score of the study population was 16.0 ± 5.7 (range 6–32). The frequency of Th17 cells in peripheral blood was mildly increased in pts with ALD in comparison with HC (1.1 ± 1.2 versus 0.9 ± 0.4; p = 0.34) without statistically significant difference. The highest level of Th17 cells was observed in Child B group, the lowest level in Child C (1.4 ± 1.6 versus 0.8 ± 0.4; p = 0.46). The mean percentage of that subset was higher in men than in women (1.23 ± 1.34 versus 0.97 ± 0.64; p = 0.93). We found no correlation of frequency of CD3+CD4+IL17+ cells to Child-Pugh and MELD scores, serum aminotransferases, alkaline phosphatase, γ-glutamyltranspeptidase, C-reactive protein, white blood cells, INR, bilirubin level.

Discussion/Conclusion: Patients with ALD tend to have mildly higher proportion of IL-17-producing cells among circulating CD3+CD4+ lymphocytes. The highest frequency was observed in Child B group, the lowest in Child C group.
Interleukin-6 genotypes play a role in susceptibility to development of chronic hepatitis C in the Turkish population

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Introduction: It is already established that host immune responses are partially under genetic control. The aim of this study was to examine the effect of functionally relevant polymorphisms in tumor necrosis factor-alpha (TNF-α) -308, interleukin-10 (IL-10) -819 and -1082, interleukin-6 (IL-6) -174, interferon-gamma (IFN-γ) +874 and susceptibility to development of chronic hepatitis.

Methods: Blood samples from 31 patients with clinically confirmed chronic hepatitis and 71 healthy controls were compared. DNA was extracted from blood using the QIAmp DNA Mini Kit (Quiagen, UK). Genotyping was carried out by ARMS-PCR technique. The frequency distribution of cytokine alleles, genotypes and haplotypes were analysed and compared using Chi-square test ($\chi^2$) in SPSS statistical analysis software.

Results: Results showed IL-6 G/C and G/G genotypes were significantly associated to susceptibility development of chronic HCV ($p < 0.05$), while no significant difference was observed for IL-6 C/C genotype. Furthermore statistical evaluation of cytokine polymorphisms of TNF-α, IL-10, and IFN-γ were seen to be statistically insignificant between chronic HCV patients and healthy controls ($p > 0.05$).

Discussion/Conclusion: In conclusion, the present study shows genotypes G/C and G/G of cytokine IL-6 at position -174 to play a role in predicting susceptibility to development of chronic hepatitis C in a Turkish population. Further studies with a larger cohort and different cytokines should be investigated to fully understand the contribution of cytokine polymorphisms to pathogenesis of chronic hepatitis C.
Genistein improves hepatic lipid metabolism in an in-vitro hepatosteatosis model

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Introduction: Genistein is suggested to improve hepatic lipid levels by increasing the transcription factor peroxisome proliferator-activated receptor type alpha (PPARα) or decreasing lipogenesis via the active transcription factor sterol regulatory element-binding protein type 1c (SREBP1c).

Our aim was to apply an in-vitro steatosis model on primary human hepatocytes to investigate the effects of Genistein on lipid metabolism as well as to identify the probable molecular targets and their respective impact on signal transduction.

Methods: Primary hepatocytes were treated with 0.5 mM oleic acid and palmitate (2:1). Genistein was applied for 24 hours up to 90 µM. Furthermore, estradiol, the estrogen receptor antagonist ICI182,780 or the MEK1/2 antagonist PD98059 were added to illustrate probable pathways. Lipid content was measured photometrically by Oil Red O staining and normalised with Sulforhodamine B staining. Cytosolic and nuclear protein of SREBP1c, PPARα and ERK1/2 as well as mRNA of SREBP1c, PPARα, fatty acid synthetase (FASN), carnitine-palmitoyl-synthase type 1 liver form (CPT1L) and long chain acyl-CoA-synthetase (ACSL) were measured using Western blot and realtime-PCR.

Results: A treatment with 60 and 90 µM Genistein significantly elevated protein levels of nuclear PPARα. Also, increased mRNA concentrations of PPARα, CPT1L and ACSL were measured. The in-vitro steatosis increased protein and mRNA of SREBP1c and mRNA of FASN, however, Genistein decreased it although not significantly relevant. PD98059 did not make a difference on SREBP1c, but it decreased PPARα. Neither ICI182,780 nor estradiol influenced the effect of Genistein on PPARα and SREBP1c.

Discussion/Conclusion: Genistein effectively lowered hepatic lipid content in steatotic primary human hepatocytes. Elevated PPARα, CPT1L and ACSL levels suggest that Genistein enforces β-oxidation by increasing PPARα levels. Also, Genistein decreased SREBP1c and fatty acid synthesis. Therefore, Genistein improves lipid accumulation of human steatotic hepatocytes and can be a valuable tool against fatty liver disease.
IL-13 induces connective tissue growth factor in rat hepatic stellate cells via TGF-β-independent Smad signaling

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Introduction: Connective tissue growth factor (CTGF) plays a central role in stimulating extracellular matrix deposition in the liver, and hence is considered as a critical mediator of TGF-β-dependent fibrogenesis. Hepatic stellate cells (HSCs) are known as the major source of CTGF. However, previous studies revealed that IL-13, rather than TGF-β, represents the main inducer of CTGF expression in HSCs.

Methods: We evaluate the effect of IL-13 on CTGF expression in primary cultured HSCs. We also investigated how IL-13 downstream signaling modulates CTGF expression in HSCs.

Results: IL-13 induces a time- and dosage-dependent increase of CTGF in a TGF-β-independent manner. This process involves different Smad proteins and their upstream receptor kinases (ALKs). Smad1 and Smad2 were identified as key mediators for IL-13 dependent CTGF expression. Furthermore, IL-13 induces Stat6 phosphorylation in HSCs, but Stat6 was not involved in CTGF induction. Instead, the Erk1/2-MAPK pathway was responsible for IL-13-induced early Smad phosphorylation and CTGF production.

Discussion/Conclusion: We demonstrate that IL-13 induces CTGF expression in HSCs by activating TGF-β-independent ALK/Smad signaling via the Erk-MAPK pathway rather than via its canonical JAK/Stat6 pathway. These results may provide an improved new insight into the molecular mechanisms of pro-fibrotic IL-13 activity in the liver.
Genistein modulates insulin-responsive pathways in a fatty liver model

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Introduction: Hepatic steatosis in the context of nonalcoholic fatty liver disease (NAFLD) is associated with insulin resistance. In our study we investigated the effects of Genistein on insulin-responsive downstream targets in an in-vitro model of hepatic steatosis.

Methods: Primary human hepatocytes were treated with oleic and palmitic acid (2:1, 0.5 mM) for 24 h to induce an intracellular fat overaccumulation. Oil Red O and SRB staining evaluated the in-vitro model of hepatic steatosis. Cells were treated with different concentrations of Genistein (7.5–90 µM) for up to 36 hours of incubation. Different experimental settings with insulin, glucose, estradiol, ICI182,780, LY294002 and PD98059 were used to demonstrate pathways and to detect possible targets of Genistein. Phosphorylation of IRS-2, Akt and ERK1/2 was detected by western blot. The mRNA expression of insulin receptor, GLUT-2, SREBP-1c, ChREBP and FOXO1 was measured by realtime-PCR.

Results: Primary human hepatocytes treated with free fatty acids revealed a decreased phosphorylation of Akt and increased mRNA expression of SREBP-1c as an evidence of impaired insulin signaling. In addition 90 µM Genistein applied for 24 hours decreased insulin induced phosphorylation of Akt but showed an increased phosphorylation of ERK1/2. Furthermore, Genistein decreased glucose output of hepatocytes via GLUT-2 transporter at high glucose concentration.

Discussion/Conclusion: Genistein impairs hepatic insulin signaling by inhibiting insulin-induced phosphorylation of Akt in a time and dose-dependent manner. Elevated activation of ERK1/2 is a possible mechanism whereby Genistein lowers the lipid content in hepatic steatosis. It is likely that Genistein modifies the carbohydrate metabolism via decreased gluconeogenesis and increased glucagon storage. Further studies are needed to elucidate the molecular mechanisms whereby Genistein affects hepatic insulin resistance.
Inverse correlation between increased intrahepatic regulatory T cells and CD56+ cells in metastatic liver of colorectal cancer patients

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Introduction: Tumor-infiltrating T lymphocytes play an important role in primary colorectal cancer, but their role in liver metastases is poorly understood. Various lymphocyte populations with predominance of cytotoxic T lymphocytes and innate lymphocytes, such as natural killer (NK) and lymphocytes called NKT were found in the human liver. Cytotoxic T cells, NK and NKT cells are effectors that realize cell-mediated cytotoxicity and tumor surveillance in the liver. CD4+CD25+FOXP3+ regulatory T cells (Treg) have been characterized as a critical population of immunosuppressive cells that maintain peripheral tolerance to self antigen and also inhibit antitumor immune response. The present study addresses the characterization of intrahepatic lymphocyte subpopulations, namely CD3+ lymphocytes, NKT lymphocytes expressing the CD3+CD56+ phenotype, CD56+ NK cells, CD4+, CD8+ T cells, and CD4+CD25+FOXP3+ Treg in metastatic liver tumors.

Methods: Eighteen colorectal cancer patients who underwent liver resection for metastatic liver tumors and 17 patients with hemangioma as controls were prospectively included. Fresh liver samples were obtained after surgical resection of metastatic or liver tissue. Phenotype characterization of intrahepatic lymphocytes (IHL) was performed using flow cytometry on intrahepatic mononuclear cells and their localization in metastatic liver tissue were examined immunohistochemically. In addition, intrahepatic Treg were identified and characterized as CD4+CD25+FOXP3+ by multicolor flowcytometric analysis.

Results: The peritumoral as well as the intratumoral inflammatory infiltrate consisted mainly in CD8+ and CD4+ cells and less of CD56+ cells (NK and NKT). Flow cytometry revealed a dominance of CD8+ lymphocytes with a lower number of CD4+ lymphocytes in metastatic, as well in non-metastatic liver. We found a significantly lower proportions of CD3+CD56+ (NKT) and CD3-CD56+ (NK) subsets in metastatic liver compared to non-metastatic liver (p < 0.05). The proportion of intrahepatic Treg was significantly higher in metastatic livers than in control livers (p < 0.01). Importantly, increased Treg infiltration in metastatic liver correlated with decreased population of NK (r = -0.796, p < 0.001) and NKT (r = -0.643, p < 0.01) cells.

Conclusion: Our data demonstrate that increased number of Tregs in liver metastases might be responsible for the suppression of innate immune system and the failure of innate antitumor response.
Plasma membrane localization of PKC-zeta is required for cell-cell adhesion but not JAK/STAT signaling in response to interferon-alpha in hepatocytes

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Introduction: Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. Pegylated interferon-alpha (IFN-α) treatment, which activates the antiviral JAK/STAT pathway, is currently the standard therapy for HCV although response rates are low (40–80% depending on genotype). Members of the protein kinase C (PKC) family of enzymes have been shown to be involved in STAT activation and PKC activity is required for reduction of HCV viral replication in response to IFN-α (using a replicon system).

Methods: Using a panel of PKC isoform-specific antibodies (a, b, bII, d, e, i and z) we investigated their expression in Huh7 whole cell fractions by Western blot analysis. Immunofluorescent antibody-specific confocal microscopy was then used to determine the subcellular localisation of these isotypes before and after IFN-α treatment.

Results: The atypical PKC-zeta (PKC-ζ) isotype was expressed at the plasma membrane/cell-cell junctions and translocated to the cytosol in response to IFN-α. Using immunofluorescence microscopy we demonstrate that PKC-ζ co-localises with the tight junction protein Claudin 1. Following IFN-α treatment cells failed to form normal cell-cell contacts. PKC-ζ was constitutively active (phosphorylated at T410) with or without IFN-α treatment but when it’s activity was blocked by a pseudosubstrate inhibitor, cell-cell contacts were disrupted in a similar manner to IFN-α treatment. Blocking PKC-ζ activity by a pseudosubstrate inhibitor did not prevent STAT1 or STAT3 activation following IFN-α treatment.

Discussion/Conclusion: These studies demonstrate that while PKC-ζ is not involved in the STAT1/3 phosphorylation in response to IFN-α, it is responsive to this cytokine and regulates cell-cell junction integrity.
Is it possible to predict the degree of fibrosis in chronic hepatitis C patients using routine blood tests in our daily practice?

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Introduction: In chronic hepatitis C infection, a liver biopsy provides important information that guides treatment decisions, but is invasive, expensive and associated with possible complications. The aim of this study was to validate and compare the diagnostic performance of two simple non-invasive fibrosis tests: the aspartate aminotransferase/platelet ratio index (APRI) and age-platelet (AP) index with the stage of liver fibrosis.

Methods: We included 110 patients (70 male and 40 female) with chronic hepatitis C and histological data of liver biopsy specimens. Staging was performed according to Ishak score. Mild fibrosis was determined with F0–F1 and advanced fibrosis with F ≥ 2. We calculated APRI and AP index.

Results: Mild fibrosis (F0–F1) was identified in 48% of the patients, while advanced fibrosis (F2–F6) was identified in 52% of the patients according to biopsy results. The area under the curve of the receiver operator characteristics (ROC AUC) of the APRI for predicting severe fibrosis was 0.72 (95% CI: 0.63–0.80; p = 0.0001). The cutoff was found at 22.62 (sensitivity of 82.5%, specificity 52.8%). ROC AUC of the AP score for advanced fibrosis was 0.78, (95% CI: 0.63–0.81; p = 0.0001), the cutoff was 29.75 (sensitivity 61.4%, specificity 84.9%). Both scores differed significantly two groups of the patients (p = 0.0001). Using multivariate logistic regression we found that platelets and AST are independent co-variants that determine the significance of the fibrosis in our patients.

Discussion/Conclusion: Both scores are equally predictive in differentiation of non-significant/significant fibrosis. APRI score showed a weaker correlation then AP score in more advanced stages of fibrosis (Ishak 5 and 6).
Expansion of myeloid-derived suppressor cells in the livers of mice with experimental autoimmune encephalomyelitis

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Introduction: Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of immunosuppressive myeloid cells and participate in the development of autoimmune disorders. MDSCs are present in the steady state liver, known for its remarkable immunoregulatory capacity, but their role in control of autoimmune diseases has not been investigated. The aim of this study was to examine hepatic CD11b⁺Gr1/Ly6G⁺ and CD11b⁺Ly6C⁺ MDSCs in experimental autoimmune encephalomyelitis (EAE). We found increased MDSC frequencies in the spleen of mice with EAE.

Methods: MDSCs were investigated by flow cytometry in livers of C57BL/6 mice (n = 5/group) that developed monophasic EAE after inoculation of myelin oligodendrocyte glycoprotein (MOG₃₅-₅₅) in complete Freund’s adjuvant (CFA) and pertussis toxin (PT) and of control naïve mice or mice immunized with CFA+PT.

Results: Hepatic non-parenchymal cells were increased (p < 0.01) in EAE-affected and CFA+PT-treated animals but these mice did not have liver injury based on blood alanine transaminase levels. Hematoxylin and eosin stained liver sections revealed remarkable polymorpho- and mononuclear cell infiltration in both treated groups. In particular, frequencies of hepatic CD11b⁺Gr1/Ly6G⁺ and CD11b⁺Ly6C⁺ cells were significantly increased in mice with EAE (p < 0.001) or treated with CFA+PT (p < 0.01). Moreover, expansion of CD11b⁺Gr1/Ly6G⁺ subsets was more significant in mice with EAE than in mice immunized with CFA+PT (p < 0.01).

Discussion/Conclusion: Treatment of mice with CFA and PT augments the proportions of granulocytic and monocytic MDSCs both in the liver and spleen. Expansion of MOG₃₅-₅₅-specific CD11b⁺Gr1/Ly6G⁺ cells, however, is a liver-specific phenomenon that might indicate the involvement of the liver in regulation the pathogenesis of EAE.
Mdr2<sup>−/−</sup> mice as model for hepatic osteodystrophy

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Introduction: People suffering from chronic liver diseases often show reduced bone mineralization, increasing their risk for fractures. Mice deficient in the ABCb4 transporter (MDR2<sup>−/−</sup>) suffer from chronic liver disease similar to PBS and PSC. Aim of this project was to examine these mice for changes in bone mineral density and architecture over time and to identify possible regulatory mechanisms.

Methods: MDR2<sup>−/−</sup> and wildtype mice (n = 4) were sacrificed 5, 11, 15, 20, 30 and 44 weeks after birth. Liver damage was analyzed by histological stainings (H&E and Masson Trichrome Goldner) as well as serum levels of LDH and transaminases. Bone mineral density and architecture was analyzed using micro-CT data. Expression of target genes was analyzed using semi-quantitative RT-PCR from liver and bone tissues and ELISA from serum.

Results: Progression of liver damage increased most significantly in MDR2<sup>−/−</sup> mice from week 5 to 15. Wildtype mice showed no liver damage at all observed time-points. Micro-CT analysis revealed that from week 20 on bone volume and trabecular number is reduced, while trabecular separation is increased in MDR2<sup>−/−</sup> mice. RT-PCR showed that the inflammatory parameters TNF-α and RANKL expression was increased while osteoprotegerin expression was decreased in livers of MDR2<sup>−/−</sup> mice. At the same time osteopontin and osteocalcin expression was significantly reduced in bone of MDR2<sup>−/−</sup> mice. Circulating active TGF-β levels decreased with age in wild-type mice, whereas MDR2<sup>−/−</sup> mice had constantly elevated (up to 120 fold at week 30) serum levels of this cytokine.

Discussion/Conclusion: Our data show that chronic liver damage in MDR2<sup>−/−</sup> mice is associated with reduced bone mineral density. As possible connection, we propose down-regulation of osteoprotegerin, which in combination with increased RANKL favors osteoclastogenesis. Furthermore, the continuously high TGF-β levels may inhibit osteoblast function as presented by down-regulation of osteopontin and osteocalcin.
Tryptophan 2,3-dioxygenase: A liver-specific enzyme with antimicrobial and immunoregulatory properties

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In mammals the regulation of local tryptophan concentrations by the interferon-gamma inducible enzyme indoleamine 2,3-dioxygenase (IDO) is a prominent antimicrobial and immunoregulatory effector mechanism. Here, we show time that another tryptophan degrading enzyme, the liver specific tryptophan 2,3-dioxygenase (TDO), is also capable of mediating antimicrobial and immunoregulatory effects.

Using a tetracycline inducible eukaryotic system, we were able to express recombinant TDO protein, which exhibits functional properties of native TDO. We found that HeLa cells expressing recombinant TDO were capable of inhibiting the growth of bacteria (Staphylococcus aureus and group B streptococci), parasites (Toxoplasma gondii) and viruses (herpes simplex virus). These TDO-mediated antimicrobial effects could be blocked by the addition of tryptophan, while the IDO specific inhibitor 1-methyl-tryptophan had no effect.

In addition we observed that, similar to IDO-positive cells, TDO-positive cells, were capable of inhibiting anti alloantigen- and CD3-driven T cell proliferation and IFN-γ production.

Here, we describe that TDO mediates antimicrobial and immunoregulatory effects and suggest that TDO-dependent inhibition of T cell growth might at least be in part responsible for the immunotolerance observed in vivo during allogeneic liver transplantation.
Darbepoetin inhibits proliferation of Huh-7 and Hep3B cells via up-regulation of the tumor-suppressor gene p53

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Introduction: Aranesp (Darbepoetin) was licensed for patients with solid tumors suffering from chemotherapy-dependent anemia in 2002. In general, a better blood supply may favor tumor growth and spreading. Thus, aim of this project was to investigate direct effects of Darbepoetin on liver tumor cell lines or primary human hepatocytes.

Methods: Primary human hepatocytes were isolated by collagenase perfusion according to the ethical guidelines of the MRI. Human hepatoma cell-lines used were HepG2, Hep3B, SkHep1, Huh-7, AKN1, HCC-T and HCC-M. Cell viability was measured by Alamar Blue conversion. LDH release, DNA laddering and FACS analysis was used to investigate cellular damage. P53 expression was investigated by Western blot analysis.

Results: All cells were stimulated with different concentrations of Darbepoetin (0, 0.15, 0.31, 0.63, 1.26, 2.5, 5, 10, 20 and 40 ng/ml). After 24 hours HepG2, SkHep1, AKN1, HCC-T and HCC-M cells showed no significant reduction in viability. Surprisingly, Huh-7 and Hep3B cells showed less Alamar Blue conversion, dose-dependently from 5 ng/ml Darbepoetin on. At those concentrations no LDH-release into the culture supernatant could be detected. Furthermore, FACS analysis and DNA laddering excluded apoptosis or necrosis as the cause for the reduced Alamar Blue conversion observed in those cells. Interestingly, Western blot analysis revealed that Darbepoetin treatment increased p53 expression in both cells, causing growth arrest as observed early in senescence.

Discussion/Conclusion: Our data show that Darbepoetin causes reduced growth in the hepatic cell lines Huh-7 and Hep3B by up-regulation of the tumor-suppressor gene p53. If we manage to further analyze the underlying mechanisms Darbepoetin might be not only useful for patients with anemia but also be applied locally to suppress growth of certain tumors.
PPAR-γ2 Pro12Ala and ACE I/D genes polymorphism contribute into immunity and metabolic disorders in obese patients with hepatic steatosis and hypertension

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Introduction: While hepatic steatosis (HS) and arterial hypertension (AH) have multiple common mechanisms of development involving metabolic and immune changes the aim of study was to investigate the influence of Pro12Ala polymorphism of PPAR-γ2 gene and I/D polymorphism of ACE gene on metabolic profile and cytokines in obese patients with HS and AH.

Methods: Study included 154 HS patients with AH (87 male, 67 female, age 50.06 ± 7.34 years). Duration of HS 1–5 years, AH 3–21 years. Metabolic disorders were defined with body mass index (BMI), glycemia, immunoreactive insulin (IRI), total cholesterol (TC), low and high density cholesterol (LDL-C, HDL-C), triglycerides (TG), C-peptide (CP) levels and HOMA-IR index. TNF-α and leptin plasma levels were assessed by ELISA. Genes' polymorphism of PPAR-γ2 (Pro12Ala), and ACE (I/D) alone or in combination was studied with PCR.

Results: Differences of BMI, plasma glucose, IRI, HOMA-IR, CP and leptin are independent from ACE gene genotypes (p > .05). Pro-allele carriers of PPAR-γ2 gene have higher BMI than AlaAla carriers (32.7 ± 2.1 and 27.9 ± 1.1 kg/m² vs 25.6 ± 0.8 kg/m², accordingly (p < .05), leptin level – 14.3 ± 0.41 and 8.6 ± 0.25 ng/ml vs 3.7 ± 0.22 ng/ml, (p < .001), glucose level – to 10.2% and 10.9% accordingly (p < .05); CP level was higher in ProPro-genotype than in Ala-allele carriers to 15.7% (p < .05). Risk group of dyslipidaemia are ProPro-genotype carriers of PPAR-γ2 gene with higher level of TC, TG and LDL-C to 16.4%, 17.3% and 27.9% (p < .05) and lower level of HDL-C in women to 25.6% (p = .038). Lipids levels are independent on ACE I/D polymorphism. Baseline TNF-α plasma levels did not significantly deviate between genotypes of PPAR-γ2 gene, but D-allele carriers (I/D+DD) of ACE gene had higher baseline TNF-α plasma levels (91.61 and 109.11 pg/ml, accordingly p < .01).

Discussion/Conclusion: In HS hypertensive patients metabolic disorders are associated with PPAR-γ2 Pro-allele (carbohydrates) and ProPro-genotype (lipids). Presence of D-allele of ACE gene is associated with reliably higher level of TNF-α plasma levels.
Endothelial function of mesenteric vessels and intestinal dysbiosis in hepatic steatosis and hypertension: chemokines are regulated by I/D ACE and A1166C AGTR1 genes polymorphisms

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Introduction: It is known that hepatic steatosis (HS) and arterial hypertension (AH) has to some extent common pathogenesis realized through metabolic and immune mechanisms involving vascular and digestive system injury. The aim is to evaluate the endothelial function and mesenteric vessels remodeling depending on I/D polymorphism of angiotensin-converting enzyme (ACE) gene and A1166C polymorphism of angiotensin II type 1 receptor (AGTR1) gene in patients with HS and AH.

Methods: Study included 104 HS patients combined with AH (50 female, 54 male, age 53.2 ± 8.7 years). Intimae-media thickness (IMT) of abdominal aorta (AO) and other flow mediated parameters of mesenteric vessels state evaluated by sonography. NO (nitrite/nitrate) plasma concentration, vascular adhesive molecule (sVCAM-1) level was defined by IEA. ACE (I/D) and AGTR1 (A1166C) genes polymorphisms assessed with PCR.

Results: High risk of endothelial dysfunction (ED) is: D-allele of ACE gene ($p < .001$); C-allele of AGTR1 gene ($p = .02$). In DD-genotype were higher flow-mediated parameters of mesenteric vessels. IMT of AO was thicker in CC-genotype of AGTR1 gene ($p = .039–.01$) and independent on I/D genotypes of ACE gene. sVCAM-1 level was higher ($p < .01$) in D-allele of ACE gene, than in II-genotype and not depending on A1166C polymorphism of AGTR1 gene. NO plasma level doesn't depend on ACE (I/D) and AGTR1 (A1166C) genes polymorphisms. Vascular remodelling in HS patients (AO IMT enlargement for more than 0.9 mm) is associated with dysbiosis III–IV grades' and sVCAM-1 plasma level growth by 1.3-1.6 times ($p < .05–.001$). AO IMT correlates with dysbiosis severity, presence of HS and sVCAM-1 level and is independent from AH severity.

Discussion/Conclusion: Risk contingents for endothelial dysfunction and mesenteric vessels remodelling in HS patients are: by sVCAM-1 plasma level and diameter of mesenteric vessels increasing – DD-genotype carriers of ACE gene; by IMT of abdominal aorta enlargement – CC-genotype carriers of AGTR1 gene ($p \leq .02–.001$).
Microbial endotoxin added to tissue damage causes "explosion" of cytokines in experimental model of liver injury

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Introduction: It is generally accepted that liver injury causes extensive immunological response characterized by significant growth of IL-1β, TNF-α, IL-6 and other proinflammatory mediators. Following idea that microbial endotoxins cause similar immune response the aim of the study is to determine the dominating etiology factor for immunologic changes following liver injury.

Methods: Liver injury was modelled intraoperatively in 87 Wistar rats under general anaesthesia; sutures were applied immediately. 45 rats (51.72%) additionally received S. typhimurium endotoxin intraperitoneally. Liquid chromatography and ELISA were used for determination of cytokines levels in liver homogenates taken 24 hrs after injury.

Results: Aseptic liver injury alone cause minor changes in cytokines levels in liver homogenates compared to control: IL-1β grew insignificantly (55.72 ± 8.30 pg/g under liver injury compared to 45.37 ± 5.82 pg/g in healthy control rats, p > .05); TNF-α – 39.26 ± 4.89 pg/g and 47.63 ± 6.47 pg/g, p > .05, respectively; gamma-interferon (γ-IFN) – 112.9 ± 7.62 pg/g and 123.9 ± 10.75 pg/g, p > .05; TGF-β1 – 204.5 ± 12.17 pg/g and 196.2 ± 6.42 pg/g, p > .06. Added endotoxin intraperitoneally caused explosive growth of cytokines in liver tissue: IL-1β increased 80% to 74.27 ± 8.09 pg/g, p < .01; TNF-α grew 45% to 56.91 ± 6.53 pg/g, p < .05; γ-IFN level (419.16 ± 30.68 pg/g) raised 3.7 times compared to liver injury without endotoxin, p < .001. In contrast, TGF-β1 was almost unchanged – 236.16 ± 25.68 pg/g, p > .05.

Discussion/Conclusion: Although liver injury is accompanied by proinflammatory tendencies, though they are generally insignificant in case of isolated aseptic and immediately cured trauma. However, this presents ideal, theoretic condition; raised endotoxin level is inevitable in case of real life trauma. Obtained data shows that better control of endotoxin influence must be ensured in order to achieve sufficient results and prevent further liver inflammation. This may be valid not only for trauma itself but any liver injury including surgery and possibly autoimmune disease.
Bacterial overgrowth syndrome effect on the course of non-alcoholic steatohepatitis

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Introduction: The number of patients with non-alcoholic steatohepatitis (NASH) and bacterial overgrowth syndrome are increasing in recent years. Bacterial toxins of pathogenic and opportunistic pathogenic intestine's microflora cause concavation of "fatty liver" to tumor necrosis factor-alpha (TNF-α). It stimulates the formation of other cytokines and inflammatory cells which leads to necrosis of hepatocytes and fibrosis. Oxidative stress starts a cascade of pathogenic processes leading to liver fibrosis and influencing the prognosis of NASH. Bacterial overgrowth syndrome may also provoke the development of spontaneous bacterial peritonitis at a subsequent stage of non-alcoholic fatty liver disease (NAFLD). There are no recommendations for pharmacotherapy of NAFLD approved by FDA at the moment. It is important to study the effect of bacterial overgrowth syndrome on the course of NASH because there are the pathogenetic relationships of steatohepatitis with the intestinal bacterial overgrowth syndrome and the malabsorption syndrome.

Methods: The study included 50 patients with NASH and bacterial overgrowth syndrome and lasted for 4 months. The mean age was 35.3 ± 1.2 years. The examination included biochemical blood test for liver enzymes (AST, ALT, ALP, GGT), total cholesterol and its fractions, total bilirubin, hepatic sonography, feces analysis on intestinal bacterial overgrowth syndrome. Patients were randomized in 2 groups. The study group received ursodesoxycholic acid (UDCA, Ursofalk®) 15 mg/kg per day and 2 mln lactobacillus 2 times per day. The control group received UDCA (Ursofalk®) 15 mg/kg per day and placebo.

Results: The study group had AST/ALT index (1.0) less than control group (1.4). The ALT level decreased by 28% in study group and by 16% in control group. The AST level decreased significantly by 9% and by 5.4% in both groups. The GGT level decreased by 52.6% and by 23.5%, respectively. Total bilirubin decrease was noted in both groups: in the first group by 18.6%, in the control group by 15.7% (p > 0.05). Total cholesterol level decreased by 14.8% compared with baseline in study group and by 10.4% in the control group mainly due to LDL cholesterol decrease by 20.2%. The control hepatic sonography showed less liver fibrosis stage in study group than in control group.

Discussion/Conclusion: The results showed the etiological and pathogenetic factors caused by the presence of intestinal bacterial overgrowth syndrome and the progression of the course of NASH. Inclusion of lactobacilli in the treatment of NASH for patients receiving UDCA normalizes the liver state markers and increases the remission period.
Polyprenols effect on inflammation and liver fibrosis

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Introduction: Polyprenols are plant analogs of endogenous lipid transport dolichol, which provide glycosylation reaction in the dolicholphosphate cycle during glycoproteins synthesis. Polyprenols pharmacological action is based on substitutionary effect where there is dolichol deficit and absence or insufficiency of dolicholphosphate cycle during chronic inflammatory and degenerative liver diseases. Experimental findings show that effects of polyprenols on the activity of the cell membrane lead to repairation of damaged cells, biosynthesis of cholesterol, membrane-bound enzymes and transformation. Moreover, polyprenols are possibly involved in the transport and redistribution of phospholipids and ubiquinone. It is important to study the effect of polyprenols on inflammatory processes in the liver which lead to fibrosis and cirrhosis.

Methods: The study included 40 patients with non-alcoholic fatty liver disease (NAFLD) and lasted for 3 months. The examination included organoleptic observations; biochemical blood parameters; total protein, albumin, bilirubin and its fractions, activity of liver enzymes (AST, ALT, ALP, GGT), cholesterol, glucose, prothrombin index and monitoring of the liver condition. Patients were randomized in 2 groups. The first group (n = 19) received ursodeoxycholic acid (UDCA) 15 mg/kg/day and polyprenols (Ropren) 3 drops 3 times per day (54 mg/day) per os. The control group (n = 21) received UDCA 15 mg/kg/day and placebo.

Results: The majority of patients had positive dynamics of clinical parameters; 72% in the first group and 64% in the control group. ALT and AST levels in the first group decreased from 146.3 U/l to 37.1 U/l and from 107.7 U/l to 14.2 U/l, respectively. The lipid profile dynamics analysis showed more intense results in patients treated with polyprenols vs. placebo. Bilirubin decrease was noted in both groups; in the first group by 31.2%, in the control group by 18.7%. Liver fibrosis signs were less stated in patients treated with UDCA and polyprenols. This was confirmed with lowering of the fibrosis index (through fibro test and indirect ultrasound elastometric study) in the group taking Ropren. The increase of active T-lymphocytes and T-helper/T-suppressor index were also observed. Indicators CD3+CD4+CD8-, CD3+CD4+CD8-, CD4+CD8+CD3- single, double positive also have higher prognostic readings in patients taking Ropren. This indicates a positive impact on the immune status, especially in immunity of the cellular chain.

Discussion/Conclusion: The results showed high hepatoprotective effect of Ropren in the treatment of patients with chronic liver damage. Including polyprenols (Ropren) with UDCA in the complex therapy of liver inflammation and NAFLD, leads to the normalization of clinical signs and positive dynamics of cytolytic enzymes activity and lipid spectra of blood. This data shows that the synergistic and cumulative therapeutic effect which can be seen is related to the additional mechanism of action of polyprenols which as a result decreases liver fibrosis.

The authors express their gratitude to Solagran Ltd. Australia for helping to organise these trials and providing Ropren.
Dll4 is required in TGF-β-mediated liver fibrogenesis

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Introduction: Mutation of Jagged-1 gene, a Notch ligand, leads to Alagille syndrome. However, patients with Alagille syndrome usually do not progress into serious liver fibrosis. In the present study, we investigated the role of Notch ligands in liver fibrogenesis.

Methods: The expression of Notch ligands, including Jagged1, Jagged2, Delta-like ligand (Dll)-1, Dll3 and Dll4, was measured in 130 patients with chronic HBV infection by immunohistochemistry. The effect of Notch ligands was as well examined in TGF-β treated hepatic stellate cells (HSCs).

Results: Immunohistochemistry analysis revealed positive expression of Jagged1, Dll3 and Dll4 in fibrotic liver tissues from chronic HBV infected patients. Distinct from Jagged1 and Dll4, which majorly located in cholangiocytes and hepatocytes, positive Dll4 demonstrated in sinusoidal cells and portal tracts. Confocal microscopy analysis confirmed these Dll4-positive cells were α-smooth muscle actin (α-SMA) positive, indicating that Dll4 was upregulated in activated HSCs or myofibroblasts in chronic HBV infected patients. Dll4 positive liver cells remarkably correlated with inflammatory grade ($r = 0.6, P < 0.001$) and fibrotic stage ($r = 0.68, P < 0.001$) in HBV infected patients. In vitro, knock-down of Dll4 by using siRNA significantly increased TGF-β induced protein expression of connective growth factor and decreased protein level of α-SMA in CFSC cells, a cell line from CCl4-induced cirrhotic rat HSCs.

Discussion/Conclusion: Dll4 plays a crucial role in liver fibrogenesis possibly via influencing TGF-β dependent activation of HSCs and extracellular matrix production.
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