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DRUG AND ALCOHOL INDUCED HEPATOTOXICITY

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To my parents for always believing in me

ABSTRACT

Drug induced hepatotoxicity is the most common reason cited for withdrawal of already approved drugs from the market and accounts for more than 50 percent of cases of acute liver failure in the United States. Ethanol (EtOH) causes a further substantial amount of liver insufficiencies world wide. The current thesis was focused on the mechanisms behind hepatotoxicity caused by these agents.

Using a rat *in vivo* model for alcoholic liver disease (ALD) it was found that cytokine and chemokine levels in blood accompanied the fluctuating levels of blood EtOH, indicating that they are directly influenced by absolute EtOH concentration. During the early phases of ALD in this model, a strong initial Th1 response was observed as revealed by increased levels of cytokine as well as transcription factor mRNAs, followed by a downregulation, whereas Th2 response was decreased by EtOH over the entire treatment period of four weeks. We found that supplementation with the antioxidant NAC to ethanol treated animals decreases severity of liver damage and somewhat decreases initial inflammatory response mediated by TNFα. NAC also diminished the ethanol-induced formation of protein adducts of lipid peroxidation products like MDA and HNE. Also, the formation of antibodies against neo-antigens formed by MDA, HNE and HER protein adducts was lowered.

In order to further study the influence of oxidative stress in ALD we utilized a transgenic mouse model overexpressing the human form of CYP2E1. Pathological changes were significantly increased after EtOH treatment, and principal component analysis showed that among parameters influencing total pathology score, para-nitrophenol activity mirroring CYP2E1 activity, had the highest impact. Analysis of 39,000 gene transcripts revealed that the expression of several genes previously known to be associated with ALD as well as several TNFα induced transcripts increased in the transgenic EtOH treated mice. We also show that cytokeratins 8 and 18, known to be of importance for formation of Mallory bodies, correlated highly to total pathology score. The results strongly support the view of an important role of oxidative stress and CYP2E1 in ethanol mediated hepatotoxicity and suggest that cytokeratin 8 can be used as an *in vivo* marker for ALD.

There are many different models available to study liver toxicity *in vitro*, with the major drawback of low predictability. We showed that the human hepatoma cell line B16A2 differentiates spontaneously after long term confluent growth, into a more mature hepatocyte like phenotype. We developed a co-culture system using human hepatoma cells and monocytes and found that, compared to single cell cultures, co-cultures showed increased cytotoxicity and elevated mitochondrial oxidative stress in response to troglitazone whereas rosiglitazone was without effect. The data underline the importance of using *in vitro* models harboring different cell types for studies of drug induced hepatotoxicity.

Keywords: Hepatotoxicity, *in vitro* models, alcoholic liver disease, CYP2E1.

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LIST OF PUBLICATIONS

I. **Butura A.**, Johansson I., Nilsson K., Wärngard L., Ingelman-Sundberg M., Schuppe-Koistinen I. Differentiation of human hepatoma cells during confluence as revealed by gene expression profiling. Biochem Pharmacol., 2004, 67, pp.1249-58.

II. Ronis M.J., **Butura A.**, Sampey BP., Shankar K., Prior R.L., Korourian S., Albano E., Ingelman-Sundberg M., Petersen D.R., Badger T.M. Effects of N-acetylcysteine on ethanol-induced hepatotoxicity in rats fed via total enteral nutrition. Free Radic Biol Med, 2005, 39, pp. 619-30.

III. Ronis, M.J., **Butura, A**., Korourian S., Shankar K., Albano E., Ingelman-Sundberg M., Badger T.M. Cytokine and chemokine expression during the development of steatohepatitis in rats fed ethanol as part of intragastric low-carbohydrate diets. Exp Biol Med., 2008 Mar, 233(3), pp. 344-355.

IV. **Butura A**., Nilsson K., Morgan K., Morgan T. R., French S. W., Johansson I., Schuppe-Koistinen I., Ingelman-Sundberg M. The impact of CYP2E1 on the development of alcoholic liver damage, studied by gene expression profiling of a transgenic mouse model. 2008, *Submitted to J. of Hepatology.*

V. Edling Y., Sivertsson L., **Butura A.**, Ingelman-Sundberg M., Ek M. Increased sensitivity for drug-induced cytotoxicity using a novel human *in vitro* co-culture model. 2008, *Submitted to Toxicology and Applied Pharmacology.*

CONTENTS

LIST OF ABBREVIATIONS

ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
CYP	Cytochrome P450
ANOVA	Analysis of variance
EtOH	Ethanol
GSH	Glutathione
GST	Glutathione-*S*-transferase
HER	Hydroxyethyl
HNE	4-Hydroxy-2,3-Nonenal
HNF	Hepatocyte nuclear factor
IFNγ	Interferon gamma
IL	Interleukin
LPS	Lipopolysaccharide
NAC	N-acetyl-L-cysteine
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
NAC	N-acetyl cysteine
NF-κB	Nuclear factor kappa B
NK	Natural killer
Nrf2	Nuclear factor-erythroid 2-related factor 2
P450	Cytochrome P450
PBMC	Peripheral blood monocytes
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
SAM	S-adenosyl-L-methionine
TGFβ	Transforming growth factor beta
STAT	Signal Transducer and Activator of Transcription
Th1	T helper 1
Th2	T helper 2
TNFα	Tumor necrosis factor alpha

1 INTRODUCTION

1.1 THE LIVER

The liver is the largest solid organ, the largest gland, and the main metabolic organ of the body. Its functions are very diverse which will be highlighted in the following passages.

The liver is made up of many different cell types. However, there are 4 main cell types which will be discussed in this thesis:

- Hepatocytes
- Endothelial cells
- Kupffer cells, which are the liver resident macrophages
- Stellate cells (Ito cells), the livers fat storing cells

Besides these, there are many other smaller populations of cells, some of which will be dealt with more closely in the chapter on immune cells in the liver, like natural killer (NK) cells and dendritic cells. Other cell types include bile duct cells, smooth muscle cells and various blood cells (Hendriks, Brouwer et al. 1990).

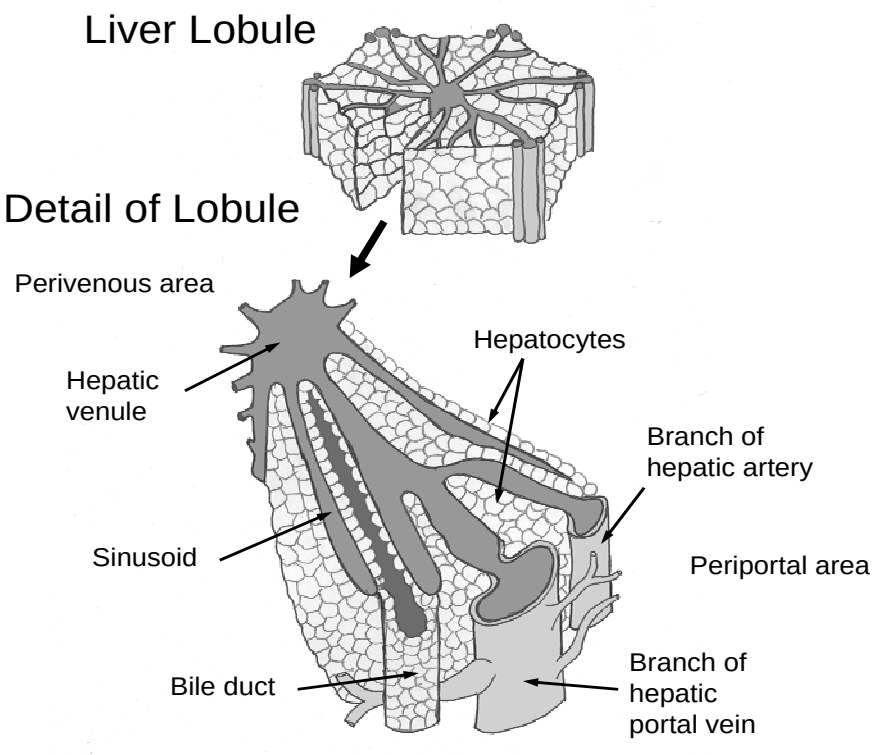


Figure 1. Schematic drawing of a liver lobule, the functional unit of the liver, with its periportal and perivenous regions. Blood flows from the portal vein through sinusoids, lined with hepatocytes to the venules in perivenous regions. Bile produced by hepatocytes flows through canaliculi and is collected in bile ducts. Adapted from Cunningham and Van Horn 2003.

1.1.1 Liver cell types

The liver is the first site of passage for venous blood arriving from the intestines via vena porta. The areas around the influx blood vessels are named periportal. The areas surrounding efflux blood vessels are the perivenous. The periportal area is highly complex and consists of a dense matrix containing collagen where afferent blood vessels are found, together with bile ducts, nerves and lymph. Spaces within the matrix contain a variable cell population, such as fibroblasts, hematopoietic cells and inflammatory cells. Also found here are epithelial cells of the bile ducts, endothelial cells of the blood vessels, and smooth muscle of arteries and veins (Grisham 1983).

The liver lobule consists mainly of plates of hepatocytes and sinusoids, with a light matrix of collagen to form a network between the two. Kupffer cells, as well as fat-storing stellate cells are found here. These types of cells reside mainly in the tissue space between the hepatocyte and the sinusoids. Terminal bile ductules connect here to the bile cannaliculi between hepatocytic plates (Grisham 1983). The walls of the hepatic sinusoid are lined by three different cell types: the sinusoidal endothelial cell, Kupffer cells, and stellate cells. Additionally, pit cells, the liver specific NK T cells are often present in the sinusoidal lumen (Kmiec 2001).

The main parenchymal mass is normally that of hepatocytes. In rat, the hepatocytes make up about 60% of liver cell count, but 70% of its mass. The remaining 40% non-parenchymal cells only make up for about 6-7% of the liver volume, while the remaining volume of approximately 23% is formed by extracellular space(Hendriks, Brouwer et al. 1990).

1.1.1.1 Hepatocytes

Hepatocytes are large, and rich in organelles such as endoplasmatic reticulum and Golgi apparatuses. They contain many and large mitochondria, as well as lysosomes and peroxisomes (Wanson, Bernaert et al. 1979; Phillips, Oda et al. 1974). A main function of hepatocytes is to participate in lipid, carbohydrate and protein metabolism. They also produce serum proteins such as albumin, coagulation factors and transferrin (Jeejeebhoy and Phillips 1976). Furthermore, hepatocytes produce and secrete bile, as well as detoxify and excrete cholesterol, steroid hormones, and xenobiotic drugs. Numerous xenobiotics are metabolized by the mixed-function mono oxidases found in hepatocytes, which will be further discussed in the chapter on the cytochrome P450´s (Sirica and Pitot 1979).

Structure and function of the hepatocytes within the liver lobule differs greatly depending on proximity to periportal or perivenous areas. Periportal type hepatocytes are often smaller, but have larger mitochondria, and a larger Golgi apparatus as compared to the perivenous type. Perivenous hepatocytes on the other hand have larger endoplasmatic reticulum. Functionally, periportal hepatocytes are more involved in gluconeogenesis, while perivenous are involved in glycolysis. Additionally, perivenous hepatocytes are dominant with respect to P450-dependent hydroxylation reactions (Smith and Wills 1981), and glutamine synthetase (Gebhardt and Mecke 1983).

1.1.1.2 Endothelial cells

The sinusoidal endothelial cells line the walls of the hepatic sinusoid and perform a function of filtration due to the presence of fenestrae. These cells also demonstrate large endocytic capacity for extracellular matrix components and immune complexes. In general they engulf smaller size particles, and may play a role in clearance of viruses, but do not possess phagocytic function in the strict sense of the word (Breiner, Schaller et al. 2001). They may also function as antigen presenting cells and secrete certain cytokines and eicosanoids (Kmiec 2001).

1.1.1.3 Kupffer cells

The liver harbors large amounts of Kupffer cells, which represent the largest tissue resident macrophage population of the body (Knook, Blansjaar et al. 1977). They are located within the sinusoid and are in constant contact with gut-derived particles that lead to a low but constant amount of activation of these monocyte derived cells. Upon activation they are able to secrete a vast range of inflammatory mediators, such as cytokines, reactive oxygen species, eicosanoids and nitric oxide (Kmiec 2001). Kupffer cells express Fc receptors that enable them to bind cells covered with immunoglobulins, or bind to complement receptors, and subsequently phagocytose cells (Smedsrod, Pertoft et al. 1985). Kupffer cells are even actively phagocytic *in vitro*, and contain high levels of peroxidase, acid phosphatase, and glucose 6-phosphate dehydrogenase (Munthe-Kaas, Berg et al. 1975; Munthe-Kaas, Berg et al. 1976). More about Kupffer cells will be discussed in the chapter on immune cells in the liver.

1.1.1.4 Stellate cells

The liver plays a central role in uptake and storage of vitamin A (retinol), and stores about 95% of retinoids found in the body (Hendriks, Verhoofstad et al. 1985; Hendriks, Brouwer et al. 1990). The fat storing perisinusoidal cells of the liver, stellate cells, are the main vitamin A storing cells. They harbor large amounts of retinol and retinyl palmitate in lipid droplets within their cell cytoplasm (Knook, Seffelaar et al. 1982). They are located in the space of Disse (between hepatocytes and sinusoid) and generally protrude to come into contact with several sinusoids (Friedman 2008). Additionally, they function to control the turnover of extracellular matrix and regulate sinusoid contractility. The stellate cells may become activated under stressful conditions and transform into myofibroblast-like cells which play a key role in inflammatory fibrotic response (Kmiec 2001). When activated, stellate cells not only proliferate, but also produce increased amount of extracellular matrix per cell. Transforming growth factor β (TGFβ) is one of the most important signals to activate stellate cells, which leads to a higher transcriptional rate of mRNAs coding for extracellular matrix components such as collagen I, fibronectin and proteoglycans. Lipid peroxidation products are also an important stimulus, whose effect may be augmented in oxidative stress conditions (Friedman 2008).

1.1.2 Liver regeneration

One of the unique features of the liver is its ability to regenerate, as first noted by the ancient Greeks in the story of Prometheus, punished by the Gods to have his liver eaten by an eagle each night, as the liver regenerated until the following night. Serial transplantation experiments have shown that hepatocytes have almost stem cell like properties, in that they have a near infinite proliferation potential (Overturf, Al-Dhalimy et al. 1999). After partial hepatectomy, the hepatocytes and cholangiocytes are first to proliferate, followed by mesenchymal cells like the endothelial cells and stellate cells. Also smaller cells named oval cells, whose functional characteristics are still controversial, can take part in regeneration (Roskams 2006). It has been shown that during liver regeneration when hepatocyte replication is delayed or impaired, the liver is instead repopulated by oval cells, considered to be a type of liver progenitor cells (Lemire, Shiojiri et al. 1991; Fausto 2005).

1.1.3 Origin of the liver

The cells that will eventually make up the adult liver originate during embryogenesis from the ventral foregut definitive endoderm (Watt, Zhao et al. 2007). The different developmental stages of the liver involve establishment of competence for liver formation, after which liver specification, hepatic bud formation, growth and finally differentiation will occur (Burke, Thowfeequ et al. 2006). During the development of the liver, as well as a certain time after partus, the metabolic profile of the young liver is far from that of the adult phenotype (Miller, Juchau et al. 1996). Prior to birth, and shortly thereafter, many metabolic changes occur in the liver (Zaret 1996). This allows the organism to adapt to uptake of nutrient from food, but also changes its ability to metabolize xenobiotics. As the organism matures, with time an adult pattern of metabolic enzymes develops. During development of hepatocellular carcinoma, frequently the gene expression pattern of the hepatocytes reverts to a more fetal-like stage (Nardone, Romano et al. 1996). In certain cases this leads to the expression of metabolic enzymes otherwise found only during embryogenesis (Karlgren, Gomez et al. 2006). This partly fetal-like expression pattern is also noticeable in many human hepatoma cell lines that are often used for *in vitro* toxicology studies. This will be partly discussed in the chapter of *in vitro* models for hepatotoxicity as well as in paper I.

1.2 THE LIVER AS A DETOXIFYING ORGAN

The adult liver is the main organ responsible for detoxifying and metabolizing a variety of exogenous as well as endogenous compounds, rendering them more hydrophilic, which often affects their potency and activity. The enzymes responsible for these actions are primarily expressed in hepatocytes and mainly divided into two groups: Phase I and Phase II.

The phase I enzymes are predominantly from the P450 family of genes, whose general function is to add polar groups, such as hydroxyl groups, to lipophilic molecules thus rendering them more hydrophilic (Park, Pirmohamed et al. 1995).

The main function of the phase II enzymes is to covalently attach a water soluble moiety to the polar group added by the phase I enzymes. Usually such molecules are sugars or peptides, such as glucuronic acid or glutathione. This usually renders the compound less reactive (Board, Blackburn et al. 1998). Examples of phase II enzymes are glutathione *S*-transferase and UDP-glucuronosyl transferase.

If the phase II reaction is impaired for some reason, or the phase I reaction is induced, this may leave the organism with an excess of reactive molecules from the phase I reaction, which can be detrimental. This can occur in the case of drug induced hepatotoxicity, when reactive metabolites of the parent compound are formed, which subsequently negatively affect cellular functions (Liu and Kaplowitz 2002).

1.2.1 The cytochrome P450´s

The cytochrome P450´s (CYP or P450) as a family have very broad, as well as overlapping, substrate specificity and are one of the largest enzyme super-families. To date about 2700 members are known, of which there are 57 putatively functional in humans (Nelson, Zeldin et al. 2004). Enzymes of the subfamilies CYP1, CYP2 and CYP3 are the primary CYP subfamilies to be involved in the metabolism of foreign compounds in mammals, with the CYP2 and CYP3 families being the predominant families involved in drug metabolism in humans (Rendic and Di Carlo 1997; Lewis, Lake et al. 2003). These families will be the ones referred to in this thesis, while families CYP4 and onwards deal mainly with endogenous substrates.

The P450´s were initially discovered about 50 years ago and are named for the absorption band at 450 nm of their reduced carbon-monoxide-bound form. It seems that they are found in most organisms, however, their amount with regard to specific isoforms found varies greatly. They are hemoproteins, generally conserved throughout evolution, the most highly conserved part being that of a heme-binding domain and their catalytic domain. Most commonly, cytochrome P450´s use electrons from NAD(P)H to catalyze activation of molecular oxygen, leading to the subsequent oxidation of their substrate. They are usually membrane proteins found in the endoplasmatic reticulum or inner mitochondrial membrane (Werck-Reichhart and Feyereisen 2000).

P450´s are involved in the biotransformation of drugs and xenobiotics, as well as in biosynthesis and degradation of many physiologically important compounds such as steroids, fatty acids, eicosanoids, fat-soluble vitamins, bile acids, etc. As the cytochrome P450´s are the main metabolizers of drugs, to determine the metabolic profile of a drug using hepatic cells rich in P450´s is crucial during drug development, and may determine if a new drug will ultimately be able to reach the market (Bernhardt 2006).

The P450´s are highly polymorphic. In some cases this results in differences in efficiency when metabolizing substrates, leading to various levels of inactivation or production of reactive intermediates of the parent compound. This is the case for several of the clinically most important hepatic P450s (Ingelman-Sundberg 2004). Interindividual differences in substrate metabolism have been proposed to be risk factors in the development of drug induced liver injury as variation in enzyme activity may lead to different levels of reactive compounds being formed (Hussaini and Farrington 2007).

1.2.1.1 CYP2E1

CYP2E1 is not one of the most important drug metabolizing CYPs in man, as it accounts for only approximately 4% of total drug oxidations known to be performed by P450s (Rendic and Di Carlo 1997). However, a large number of carcinogens and other toxicants are metabolized by CYP2E1 (Guengerich, Kim et al. 1991; Johansson, Ekstrom et al. 1988; Ingelman-Sundberg, Johansson et al. 1993). Some examples of known substrates are ethanol, acetone, toluene, benzene, carbon tetrachloride and halothane. Substrates are generally low molecular compounds and often solvents. Among commonly used drugs metabolized by CYP2E1, the most well-known and well studied are probably paracetamol and salicylic acid (Johansson and Ingelman-Sundberg 1985; Lewis, Lake et al. 2003). The metabolism of 4-nitro phenol, also known as para-nitrophenol, is often used as a measure of CYP2E1 activity (Wolf, Wood et al. 2004). Chlorzoxazone may also be used (Marchand, Wilkinson et al. 1999; Yoo, Guengerich et al. 1988).

There are also physiological roles of CYP2E1, such as fatty acid oxidation and glukoneogenesis from ketones (Lieber 2004). CYP2E1 is relatively well conserved among species. There are orthologous enzymes in rats and mice, although with minor species differences in relation to substrate specificity for compounds metabolized by CYP2E1, while the main differences are seen on a regulatory level (Lewis, Bird et al. 2000).

It has been shown that substrates such as those mentioned here, like acetone and ethanol, induce and stabilize the CYP2E1 enzyme (Eliasson, Johansson et al. 1988; Johansson, Ekstrom et al. 1988). Also endogenous substrates within lipid and ketone body metabolism may induce CYP2E1 (Lieber 2004). Alcoholics are extra susceptible to certain xenobiotics such as paracetamol due to the induction of CYP2E1 (Lieber 1988). Induction proceeds via a post-transcriptional mechanism of protein stabilization. This has been shown both *in vitro* (Eliasson, Johansson et al. 1988; Eliasson, Johansson et al. 1990), and *in vivo* by labeling of proteins with ^{14}C-sodium bicarbonate in rats, where it was shown that the usually more rapid turnover of the CYP2E1 enzyme is slowed down in the presence of substrate (Song, Veech et al. 1989). There is also some evidence that CYP2E1 induction may occur at an mRNA level, in particular under conditions mimicking diabetes mellitus (Song, Matsunaga et al. 1987; Johansson, Ekstrom et al. 1988; Woodcroft, Hafner et al. 2002). One putative mechanism may be that the peroxisome proliferator-activated receptor α (PPARα) coactivator 1α, which is induced under glucose deprivation, (Lin, Handschin et al. 2005) potentiates CYP2E1

transcription. The localization of CYP2E1 is predominantly perivenous, particularly in the induced state, and associated with a proliferation of the endoplasmatic reticulum in hepatocytes. The localization of the CYP2E1 enzyme may explain why compounds metabolized by the enzyme primarily damage the perivenous region (Ingelman-Sundberg, Johansson et al. 1988).

The transcription of CYP2E1 has been shown to be governed mainly by the liver enriched transcription factor hepatocyte nuclear factor 1α (HNF1α), as has been seen by *in vitro* transfection studies using reporter gene constructs (Liu and Gonzalez 1995), and by low levels of CYP2E1 in HNF1α knock-out mice (Cheung, Akiyama et al. 2003). However, lately it has been shown that CYP2E1 expression not only relies on HNF1α, but also on β-catenin (Sekine, Lan et al. 2006). Other unknown factors may also help regulate its expression.

The structure of CYP2E1 has not yet been fully determined as it has not been crystallized; however attempts have been made to elucidate its structure through modeling after the known CYP2C5 crystallographic template (Lewis, Lake et al. 2003). Its active site residues have however been determined, and are proposed to be the Leu363, Val364, and Leu368. Additionally, a series of phenylalanines, in particular Phe106, seem to mediate contact with the substrate (Collom, Jamakhandi et al. 2007).

In humans, a small number of allelic variants exist, however the influence of these variants on catalytic activity is not very large in comparison to the wild type (Hu, Oscarson et al. 1997; Ingelman-Sundberg 2001). There are no reports of CYP2E1 variants or polymorphisms that lead to an inactive enzyme in humans, or in animals. This, together with the enzyme being well-conserved throughout species would suggest that CYP2E1 has important functions (Hu, Oscarson et al. 1997; Hu, Hakkola et al. 1999; Gonzalez 2007).

1.3 THE LIVER AS AN IMMUNOLOGIC ORGAN

The liver is the main hematopoietic organ during certain stages in fetal development and continues to be a hematopoietic organ even after birth. It can produce all leukocytes lineages from resident hematopoietic stem cells (Abo, Watanabe et al. 1994; Kenai, Matsuzaki et al. 1995; Taniguchi, Toyoshima et al. 1996).

1.3.1 Th1 and Th2 response – brief summary

CD4+ T cells are heterogenous and can be mainly divided into two groups, T helper 1 (Th1) and T helper 2 (Th2) , on the basis of their cytokine production (Mosmann, Cherwinski et al. 1986). It is highly debated which of the two types mainly influence various types of disease. However, it has been shown that T helper 1 response is generally defined as one which generates significant amounts of interferon γ (IFNγ), which activate immune responses and dominate in several types of infection, particularly bacterial and viral (Brady, Mahon et al. 1998; Del Rio, Buendia et al. 2001; Mizuno, Takada et al. 2003). T helper 2 cells and cytokines are responsible for

transplantation tolerance (Lowry, Takeuchi et al. 1993), and allergies (Robinson, Hamid et al. 1992).

Th1 type cytokines are for example: Interleukin-2 (IL-2), IL-12, IFNγ, and tumor necrosis factor β (TNFβ). They stimulate functions of macrophage activation, opsonization and complement, and thus can be seen as primarily responsible for phagocyte dependent response/innate immunity (Romagnani 1995). IL-12 and IFNγ have been shown to drive differentiation of immune cells towards the Th1 fate (Wenner, Guler et al. 1996), via transcription factors from the family of signal transducers and activators of transcription (STATs), more specifically STAT 4 and STAT 1. Part of the mechanism is by way of IFNγ and STAT1 induction of T-bet, which in turn induces IL-12 (Afkarian, Sedy et al. 2002).

Th2 type cytokines are among others: IL-4, IL-5, IL-10 and IL-13. They induce antibody synthesis and thus can be seen as helpers of the adaptive immune response (Romagnani 1996). Furthermore, certain Th2 type cytokines such as IL-10 and IL-13 may inhibit Th1 and macrophage function, partially by inhibiting IL-12 (Aste-Amezaga, Ma et al. 1998). Th2 cytokines may induce themselves as IL-4 drives Th0 type cells to the Th2 fate (Kopf, Le Gros et al. 1993), via the transcription factor STAT 6 (Murphy, Ouyang et al. 2000).

1.3.2 Immune cells in the liver

Like previously mentioned, the portal tract of the liver contains many different cells of hematopoietic origin, as well as hematopoietic stem cells (Grisham 1983). The liver contains cells involved in both adaptive, as well as innate immunity.

1.3.2.1 Innate immunity

In comparison to other organs, the liver is particularly rich in cells of the innate immune system. The main cell types here are here Kupffer cells, NK cells, NK T cells. NK T cells are not strictly part of innate immunity but functionally somewhere in between adaptive and innate. Since they are T cells they will be dealt with under adaptive immunity. Of hepatic lymphocytes, approximately 30% are NK cells and about the same amount NK T cells, in total about 60%, indicating the great contribution of NK cells to liver immunity. This may be compared to the approximately 15% that the two cell types combined contribute to in peripheral blood lymphocytes (Norris, Collins et al. 1998; Mehal, Azzaroli et al. 2001).

NK cells are one of the main producers of liver INFγ in response to lipopolysaccharide (LPS) (Cowdery, Chace et al. 1996), which partly depends on the activation of NK cells by IL-12 derived from activated Kupffer cells (Seki, Osada et al. 1998; Seki, Habu et al. 2000). They show high cytotoxic activity towards tumor cells, with the help of the TRAIL-ligand, which is up regulated by IL-2 (Ishiyama, Ohdan et al. 2006).

Kupffer cells are derived from circulating monocytes, and play a particularly important role in initiating inflammation in the liver. Kupffer cells differ in properties depending if they are periportal or perivenous. Periportal cells are larger and more active in phagocytosis, mirroring their function as the first line of defense of the body towards gut derived bacteria entering the blood stream and reaching the liver via the vena porta (Gregory, Barczynski et al. 1992; Itoh, Okanoue et al. 1992; Sleyster and Knook 1982). Perivenous Kupffer cells on the other hand, are smaller and produce larger amounts of n itric oxide, as well as prostaglandins (Mustafa and Olson 1998) Ex vivo cultures of Kupffer cells have shown that the perivenous type secretes more than double the amount of TNFα upon LPS stimulation (Bykov, Ylipaasto et al. 2003).

Kupffer cells are one of the main cell types to secrete cytokines, which then regulate the function not only of the Kupffer cells themselves but also that of other cell types such as the NK cells. Stimulation of Kupffer cells by bacteria and bacterial LPS leads to production of IL-12 (Takahashi, Ogasawara et al. 1996), as well as TNFα (Sakurai, Terada et al. 2002; Seki, Habu et al. 2000). Other cytokines known to be produced by Kupffer cells upon LPS stimulation include IL-6, TGFβ, IL-1β and IL-8 (Kamimura and Tsukamoto 1995; Novobrantseva, Majeau et al. 2005). Cytokines derived from Kupffer cells have in turn been proven to stimulate hepatocytes to further increase chemotactic response by secretion of IL-8 (Thornton, Ham et al. 1991).

Thus, Kupffer cells and NK cells hence mainly secrete Th1 type cytokines that activate the immune system (Seki, Osada et al. 1998). Immunosuppressive cytokines such as IL-10 may instead be secreted by stellate cells (Lee, Yu et al. 2005) and regulatory T cells (but also by Kupffer cells and NK cells) (Erhardt, Biburger et al. 2007).

1.3.2.2 Adaptive immunity

Adaptive immunity can be classified into humoral immunity and cell-mediated immunity, mediated principally by B and T lymphocytes, respectively.

T cells promote differentiation of B cells to antibody secreting plasma cells. T cells kill infected cells and secrete cytokines such as TNFα, IFNγ and IL-6 (Canning, O'Brien et al. 2006; Cao, Batey et al. 1998). TNFα derived from Kupffer cells play an important role in stimulating activation of T cells which then elicit a cytotoxic response (Mizuhara, O'Neill et al. 1994; Schumann, Wolf et al. 2000). Intrahepatic accumulation of highly activated CD8+ T cells is part of the pathogenesis process in hepatitis, including alcoholic hepatitis (Bowen, Warren et al. 2002).

NK T cells constitute a distinct subpopulation of T cells that is particularly abundant in the liver, as previously mentioned. In fact, they are not strictly a part of adaptive immune response, but can be seen as having a function in between adaptive and innate immunity. These cells produce large amounts of the Th2 cytokine IL-4 (Yoshimoto and Paul 1994) but also the Th1 cytokine IFNγ (Burdin, Brossay et al. 1998). This ability of secreting both Th1 and Th2 type cytokines is a particular feature of NK cells. NK T cells are often present in the lumen of the sinusoid. They exhibit MHC-unrestricted killing of a variety of tumor cells, an activity which is enhanced by IFNγ (Kmiec 2001).

NK T cells have been shown to "crawl" within the hepatic sinusoid, and stop upon T cell antigen receptor activation (Geissmann, Cameron et al. 2005).
Naïve CD8+ T cells are also known to accumulate in the liver, where they may be activated, but at times to a lesser degree than in lymph nodes. Thus, low-grade activation of T cells in the liver rather leads to tolerance (Bowen, McCaughan et al. 2005). There is also evidence for regulatory T cells expressing IL-10 (Erhardt, Biburger et al. 2007).

B cells have not been well studied in adult liver, however, there is a substantial B cell population showing similarities to splenic B cells. It has been shown that B cells play a role in liver fibrosis, as B cell deficient mice show significantly less fibrotic lesions after carbon tetrachloride induced liver injury. This effect is independent of antibody production (Novobrantseva, Majeau et al. 2005). But also B cell antibody-dependent responses play a part in liver injury, as antibody production has been shown to be of importance in alcohol induced liver damage, which will be discussed in the chapter on alcohol hepatotoxicity (Albano, French et al. 1999).

1.4 LIVER TOXICITY

1.4.1 Drug induced hepatotoxicity

Drug induced liver injury is a health problem, and is expected to increase as the number of drugs being consumed increases, both prescription and non-prescription, as well as due to the current trend of usage of pharmacologically active substances in complementary and alternative medicine (Schoepfer, Engel et al. 2007). Drug induced hepatotoxicity is the most common reason cited for withdrawal of already approved drugs from the market. It also accounts for more than 50 percent of cases of acute liver failure in the United States. The exact incidence of drug induced liver injury is difficult to estimate, and in general, studies aiming at measuring its incidence suffer from drawbacks such as under-reporting and that data in general come from retrospective studies. Often, there is also a lack of information about self medication and usage of herbal products that may interact with prescription and non-prescription drugs (Almdal and Sorensen 1991; Garcia Rodriguez, Ruigomez et al. 1997; de Abajo, Montero et al. 2004). However there is one prospective community-based study performed in France where, over a 3 year period, the annual incidence of hepatic drug reaction was found to be 14 cases per 100.000 inhabitants (Sgro, Clinard et al. 2002). Others have reported about 0.7 to 1.3 cases per 100.000 inhabitants per year (Hussaini and Farrington 2007). Despite the frequency of drug-induced liver injury being low, data from the Centers for Disease Control and Prevention in the U.S. report approximately 1600 new acute cases of liver failure annually, of which paracetamol hepatotoxicity accounts for approximately 41% (Norris W 2008). When looking at hospitalized patients, the incidence of adverse drug reactions is estimated to be 6.7%, and fatal adverse drug reactions amount to 0.32%, as determined by a meta-analysis of about 40 prospective studies (Lazarou, Pomeranz et al. 1998). During the period 1995 to 2005, the reports of adverse drug reactions as well as deaths related to these, have more than doubled (Moore, Cohen et al. 2007).

Many cases of drug induced liver injury are idiosyncratic, *i.e.* the reaction is unpredictable based on the known pharmacological properties of the substance, and hence is easily missed during pre-clinical stages of development. There are however studies to indicate that these reactions may be dependant on an increased sensitivity of the patient to the drug in question, depending on such factors as other concomitant diseases or other concomitant drugs (Bonfanti, Landonio et al. 2001). Certain genetic factors, such as HLA-type, can in some cases contribute to the sensitivity of an individual to adverse drug reactions (Kindmark, Jawaid et al. 2007; Mallal, Phillips et al. 2008; Mallal, Nolan et al. 2002; Hetherington, Hughes et al. 2002). Commonly, clinically overt adverse drug reactions occur only after some period of latency, anywhere in the span ranging from one to 12 months (most commonly within 90 days), and nearly always disappear after removal of the drug - characteristics which are typical of hypersensitivity reactions. Drug induced liver injury may present with several different clinical features; hepatitic/hepatocellular, cholestatic or mixed (Liu and Kaplowitz 2002; Hussaini and Farrington 2007). They often have the basis in an adverse immune response, for instance towards reactive metabolites of the drug that may bind to cellular proteins and macromolecules, and hence form neo-antigens, that are recognized by the immune system. Reactive metabolites may also have a direct detrimental effect on cellular function, many times affecting mitochondrial function (Liu and Kaplowitz 2002).

Liver injury and finally cell death may be triggered by a variety of different insults. The hepatocyte mitochondria play a major role of determining the fate of an injured cell. Selective release of mediators while mitochondrial function is still intact drives the cell preferably towards apoptosis, while a profound loss of mitochondrial function is more likely to lead to necrotic cell death (Kaplowitz 2000).

Regardless of their etiology, drug induced hepatotoxicity remains a major problem during drug development in the pharmaceutical industry, both with regard to increased risk for patients undergoing clinical trials, and also patient-risk after the launch of a new drug to the market. Also, because of the increased costs that follow failure of a drug-to-be at a late stage in drug development or after its launch.

1.4.1.1 In vitro models for hepatotoxicity

Historically, almost 40 years ago, the toxicity of compounds was tested using the lymphocyte proliferation test, where a compound was added to the separated mononuclear cells of a patient, and cultured in the presence of the suspected compound. The cells were observed to determine if they transformed into a larger and more granular phenotype (Woodward and Neuberger 1997). About 30 years ago, testing on hepatic cell cultures gained popularity (Grisham 1979). Today, a large variety of methods to assess toxicity *in vitro* exist. Most commonly, these deal with adding compound to primary hepatocytes, hepatoma cell lines, liver slices or isolated perfused livers of rodents (Dambach, Andrews et al. 2005; Yokoyama, Horie et al. 1995; Thompson, Valentine et al. 2002; Gao, Ann Garulacan et al. 2004; Yokoyama, Horie et al. 2006). The drawback of such systems is that they generally offer a relatively low

level of prediction for drug toxicity due to the lack of drug metabolizing enzymes (Groneberg, Grosse-Siestrup et al. 2002). This is partially due to the problem that primary hepatocytes rapidly lose their phenotype *in vitro*, and that hepatoma cell lines have a rather de-differentiated phenotype (Li, Ralphs et al. 2007). However, there are some novel cell lines that have proven to retain relatively large amounts of drug metabolizing enzymes, such as HepaRG which will be dealt with more in the discussion (Kanebratt and Andersson 2008).

During the last 10 years, advances in gene technology have led to the birth of the toxicogenomics field, where global gene expression analyses of cells/tissues after treatment with different known hepatotoxins are used to compile large databases of patterns of gene expression. New compounds are then tested in the system used, their pattern analyzed and compared to the database (Steiner, Suter et al. 2004; Luhe, Suter et al. 2005). Also in biomechanics, advances ha ve been made to introduce bioreactors in large-scare or micro format to more efficiently conduct *in vitro* testing. The aim of these is to mimic the structures of an organ *in vivo*, and in a future perspective even the interactions of several organs *in vivo* in order to partially reduce the amount of *in vivo* testing necessary. Bioreactors and similar systems generally consist of separated cell culture chambers, interconnected by passages to allow for a continuous flow of nutrients and supplements to cultured cells, and efficient waste removal (Baudoin, Corlu et al. 2007).

The liver is made up of many different cell types, as previously mentioned. Hence systems making use of more than one cell type should be developed. Certain models of co-cultures have already been developed. For example systems where human hepatocytes are being co-cultured with stellate cells (Nieto and Cederbaum 2003), or a rat model of hepatocytes and macrophages (Tukov, Maddox et al. 2006). Also, a system of hepatic macrophages co-cultured with cholangiocytes has been developed (Alabraba, Lai et al. 2008). These studies have all been able to show mechanisms of toxicity interactions which may not have been achieved using one cell type only.

1.4.2 Alcohol hepatotoxicity

Alcohol is one of the main causes of end-stage liver disease worldwide. In the United States, alcoholic liver disease is the second most common reason for liver transplantation (Mandayam, Jamal et al. 2004). The Dionysos Study, a cohort study of the prevalence of chronic liver disease in an Italian population, showed that 21% of the population studied were at risk for developing liver damage. Of these, only 5.5% of the individuals at risk showed actual signs of liver damage (Bellentani, Saccoccio et al. 1997).
About 50 years ago it was believed that alcohol in itself was not toxic, rather that the nutritional deficiencies often accompanying it were the actual causes of liver damage. However, it was shown by Lieber and De Carli that in rats, alcoholic liver damage developed despite sufficient nutrition (DeCarli and Lieber 1967). The toxicity of alcohol was later on shown to be related to its metabolism by alcohol dehydrogenases (ADHs) and also to the metabolism by CYP2E1. There is also a component of

metabolism by catalase (Zima, Fialova et al. 2001). The main pathway for ethanol (EtOH) oxidation in the liver is via ADH to acetaldehyde, which is associated with the reduction of NAD to NADH. NADH in turn increases xanthine oxidase activity, which elevates production of superoxide (Zima, Fialova et al. 2001). Metabolism of EtOH by alcohol dehydrogenase influences the redox status of the liver also in other ways. Enhanced acetaldehyde production after EtOH metabolism decreases hepatic glutathione (GSH) content. The decrease in GSH is both due to an increased loss, as well as a lower rate of synthesis (Speisky, MacDonald et al. 1985). The absolute majority of EtOH oxidation is by ADH to acetaldehyde and further by aldehyde dehydrogenase to acetic acid. However, there is a slight portion of P450 dependent inducible EtOH oxidation due to the CYP2E1 component. Also, CYP1A2, CYP3A4 and CYP2B families may contribute to EtOH oxidation (Johansson, Ekstrom et al. 1988; Lieber 2004).

The first indications that not only alcohol dehydrogenases participate in the metabolism of ethanol came in the early 1970´s, when it was discovered that microsomal membrane fractions were capable of catalyzing the oxidation of ethanol. These reactions required NADPH, and were inhibited by CO, properties that are distinct from those of alcohol dehydrogenases (Lieber, Rubin et al. 1970). It was then discovered that this activity was due to CYP2E1 and that the enzyme was inducible by ethanol in rats (Ryan, Koop et al. 1986; Johansson, Ekstrom et al. 1988).

Free radicals have been implicated in alcoholic liver disease in various ways. Mechanisms that are thought to be involved are impairment of antioxidant defenses, as well as production of reactive oxygen species by the mitochondria and the CYP2E1 enzyme, and by activated phagocytic cells. Oxidative compounds then may lead to activation of immune cells to express pro-fibrotic and pro-inflammatory cytokines. Macrophages produce TNFα in various conditions that cause oxidative stress (Ahmed, Aronson et al. 2000), as well as IL-1 and IL-6 (Meng and Lowell 1997). Also, oxidative stress leads to the generation of lipid peroxidation products and protein adducts (Ekstrom and Ingelman-Sundberg 1989; Dupont, Lucas et al. 1998; Johansson and Ingelman-Sundberg 1985; Albano, French et al. 1999), which eventually stimulate a break in self-tolerance and an immune reaction associated with hepatitis (Albano 2006). CYP2E1 inhibitors have been shown to reduce the formation of lipid peroxidation products (Ingelman-Sundberg, Johansson et al. 1993). CYP2E1 has also been shown to be elevated in non-alcoholic steatohepatitis (Weltman, Farrell et al. 1998). Furthermore it has been shown that the increase in cellular oxidative stress by even moderate alcohol consumption may be one of the mechanisms by which alcoholism worsens progression of chronic hepatitis C, as antibodies towards albumin adducted with lipid peroxidation products were greater in consumers (Rigamonti, Mottaran et al. 2003).

It was suggested that CYP2E1 may play a role in alcoholic liver damage as it has been shown that during ethanol oxidation, CYP2E1 produces $O_2^{\cdot-}$ and H_2O_2 as a result of uncoupling of oxygen consumption with NADPH oxidation (Ingelman-Sundberg and Johansson 1984). In the presence of iron catalysts, even more reactive oxygen species (ROS) can be formed, such as the hydroxyl radical, superoxide and hydrogen peroxide (Ingelman-Sundberg and Johansson 1984; Gonzalez 2005; Cederbaum 2006; Ekstrom

and Ingelman-Sundberg 1989); Koch, Galeotti et al. 1991). These reactive oxygen species (ROS) may in turn lead to elevated levels of lipid peroxidation products, that in turn form adducts with cellular proteins and hence limit their function. Adducts may also be formed with nucleic acids. Eventually, this will result in cell damage (Cederbaum 2006). It has been shown that the various lipid peroxidation products formed lead to an immune response in human alcoholics, which worsens as disease progresses (Mottaran, Stewart et al. 2002). Lipid peroxidation products have also been shown to play a part in fibrosis, by being able to activate the stellate cells of the liver to increase their production of collagen, which has been particularly studied for the lipid aldehyde malondialdehyde (MDA) (Maher, Tzagarakis et al. 1994).

Liver injury is often zonated within the liver. This is the case for many hepatotoxins that primarily damage the perivenous region, and also true in the case of ethanol. Notably, CYP2E1 is particularly induced in perivenous regions by ethanol (Johansson, Lindros et al. 1990; Fang, Lindros et al. 1998). In vitro studies using HepG2 cells transfected to express CYP2E1, both stably and transiently, have show signs of DNA fragmentation and apoptotic cell death in transfected cells, which is not seen in controls. EtOH-induced apoptosis was prevented by an inhibitor of ethanol oxidation via CYP2E1, and also by trolox, an antioxidant that prevents lipid peroxidation (Wu and Cederbaum 1999). In this cell model, it was also noted that glutathione levels were lowered (Wu and Cederbaum 2004).

The liver however has many mechanisms to scavenge oxygen radicals, like superoxide dismutase, catalase, and glutathione peroxidase. Glutathione is undoubtedly one of the most important antioxidants, and the liver is the body's main glutathione reservoir (Franco, Schoneveld et al. 2007). The nuclear factor-erythroid 2-related factor 2 (Nrf2) has been shown to drive the transcription of several antioxidant genes such as NADPH-quinone oxidoreductase 1, glutamate cystein ligase, and heme oxygenase 1 (Lickteig, Fisher et al. 2007).

Another part of the development of alcoholic liver damage is the activation of macrophages not by oxidative stress but also by increased circulatory levels of LPS. This is by many considered to be the main pathway of macrophage activation. Macrophages are activated by LPS by way of a cascade of tyrosine phosphorylations (Weinstein, Gold et al. 1991), which in turn activates map kinases within the macrophage (Weinstein, Sanghera et al. 1992). The activation of the transcription factor AP-1 plays part in these activating mechanisms, which finally lead to the expression of inflammatory mediators (Hambleton, Weinstein et al. 1996). Cytokines such as TNFα, IFNγ and IL-1 have been shown to be released after LPS stimuli. (Tengku-Muhammad, Hughes et al. 2000). The transcription factor Nrf2 has been shown to be up regulated by LPS (Nilsson, Bajic et al. 2006), indicating that protection against oxidative stress is a major task of hepatic response to LPS.

1.4.2.1 Alcoholic liver damage – clinical studies

Although alcohol is undoubtedly a hepatotoxin, still only approximately 20% of heavy drinkers (males) develop alcoholic cirrhosis (Lelbach 1975). Cirrhosis is most often preceded by hepatitis, generally defined by the following morphologic features; hepatocyte necrosis, neutrophil polymorph infiltrate, fatty changes and usually also Mallory bodies (accumulations of hyaline material in damaged hepatocytes) (Beckett, Livingstone et al. 1961). The series of events preceding cirrhosis are commonly: fatty accumulation, hepatitis, fibrosis and finally cirrhosis.

Immunological reactions play a large role in alcoholic liver disease. It has long been known that chronic alcoholic liver disease as well as hepatitis is associated with elevated serum cytokine levels, which have prognostic value. TNFα is associated with increased mortality in alcoholics (Yin, Wheeler et al. 1999). Serum levels of TNFα, TNF receptor 2 and IL-8 have been shown to be significantly elevated in alcoholics (Gonzalez-Reimers, Garcia-Valdecasas-Campelo et al. 2007). Liver disease patients have also been shown to have elevated levels of the pro-inflammatory cytokines IL-6 and IL-8 (Gonzalez-Quintela, Vidal et al. 1999) as well as TNFα and IL-2. IL-6 and IL-8 are also elevated in alcoholic patients without liver disease. Interestingly, also levels of anti-inflammatory cytokines such as IL-10 are elevated in alcoholics with liver disease/cirrhosis (Latvala, Hietala et al. 2005; Szuster-Ciesielska, Daniluk et al. 2000). Clinically, dietary supplementation with SAM to restore glutathione levels, and phosphatidylcholines to restore membrane function, have been successful and proved some favorable effects on parameters of liver damage (Lieber 2005). S-Adenosyl methionine (SAM) is a precursor of glutathione. The drug N-acetylcysteine (NAC), also a glutathione precursor, is used clinically to prevent hepatic failure in the case of paracetamol overdose (Lauterburg, Corcoran et al. 1983; Shah and Gordon 2007). The beneficial effect of SAM has also been shown in various animal models. In rats exposed to ethanol and LPS, SAM treated animals showed significantly decreased fibrosis, oxidative stress and steatosis. Supplementation also improves liver function. SAM also decreases as TGFβ mRNA levels and stellate cell activation (*Karaa* 2008). SAM has also been proved to attenuate EtOH induced glutathione depletion and associated mitochondrial lesions in baboons (Lieber 1994).

1.4.2.1.1 T cells and innate immunity

It has been shown that in chronic alcoholics, even before the development of liver disease, there is an increased level of both CD4+ and CD8+ peripheral blood T cells, as well as of IFNγ and IL-2 levels(Laso, Iglesias-Osma et al. 1999). Significant peripheral blood mononuclear cell T cell proliferation can also be induced by MDA adducted to serum albumin in patients with alcoholic liver disease, but not in controls (Stewart, Vidali et al. 2004). However patients after 1 year of withdrawal showed decreased expression of Th1 type cytokines, down to levels of controls. Interestingly, a marked increase in IL-4, a Th2 type cytokine, has also been noted. Still, when the ratio of IFNγ producing cells is compared to that of IL-4+ lymphocytes, there is a predominance of IFNγ producing cells (Laso, Iglesias-Osma et al. 1999). This imbalance with Th1

response dominating over Th2 has been confirmed by others (Dominguez-Santalla, Vidal et al. 2001).

Also peripheral blood dendritic cells are affected by EtOH treatment. Spontaneous secretion of TNFα, IL-1β, IL-6 and IL-12 *ex vivo* has been shown to be increased in dendritic cells of chronic alcoholics even without development of liver disease. After withdrawal, the number of total circulating dendritic cells decreases (Laso, Vaquero et al. 2007). With regard to NK cells, there have been reports of a paradoxically decreased NK cell activity in alcoholics (Charpentier, Franco et al. 1984). NK cell production of TNFα was reduced in alcoholics without liver disease in comparison to controls after stimulation of NK cells with IFNα and IL-12. In alcoholics with liver cirrhosis, TNFα production varied depending on EtOH intake status at time of evaluation, being higher when there was an active EtOH intake (Laso, Lapena et al. 1997).

In alcoholic hepatitis patients, a significant increase in activated T cells and NK cells is seen in peripheral blood as well as in the liver. These changes remain for several months after alcohol withdrawal (Laso, Madruga et al. 1997).

1.4.2.1.2 B cells and antibodies

Serum from heavy drinkers contains IgG, IgM and IgA antibodies towards acetaldehyde-derived protein adducts, which are significantly elevated in heavy drinkers in comparison to patients with non-alcoholic liver damage and controls. The total anti-adduct immunoglobulin amount has been shown to correlate to the severity of disease (Viitala, Israel et al. 1997). Similar patterns have been seen with regard to antibodies directed specifically towards MDA-protein adducts, where antibody titers also correlate to disease severity (Viitala, Makkonen et al. 2000). However, antibodies directed towards human serum albumin adducted with MDA have also been detected in non-alcoholic fatty liver disease as it progresses towards fibrosis (Albano, Mottaran et al. 2005). The sites of CYP2E1 expression co-localizes with sites of acetaldehyde- and MDA-adducts in the livers of alcoholic patients (Niemela, Parkkila et al. 2000). It has also been shown that 4-hydroxy-2,3-nonenal (HNE) adducted to albumin induces antibody formation in alcoholics (Mottaran, Stewart et al. 2002), and that IgG autoantibodies towards CYP2E1 develop in patients with alcoholic liver disease (Vidali, Stewart et al. 2003; Clot, Albano et al. 1996).

1.4.2.2 Animal models of alcoholic liver damage

As previously mentioned, liver damage resulting in cirrhosis is a development in several steps, fatty liver being one of the first steps. These intricate steps are difficult to model and to this day no available animal model can be said to fully mirror the pathogenesis process in humans. However, there are several major breakthroughs in animal models of alcoholic liver disease using rodents.

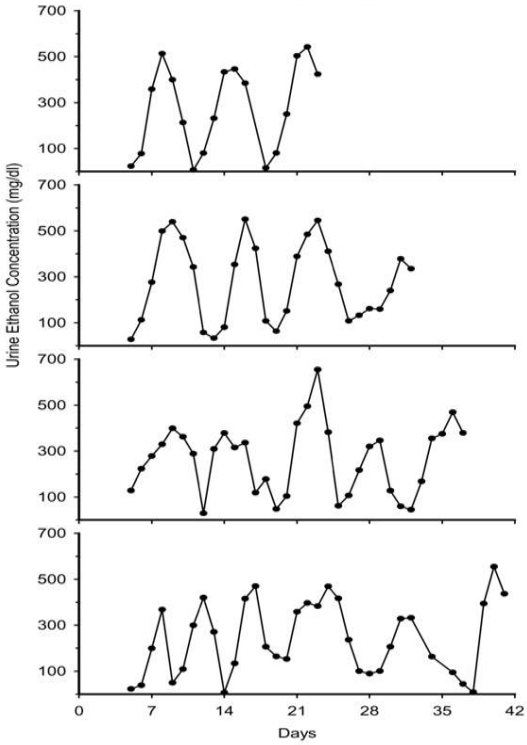
1.4.2.2.1 Liber-deCarli model

One of the first successful rodent models of alcoholic liver disease (ALD) was that of Lieber and de Carli (DeCarli and Lieber 1967). The development of the model was driven by the aversion of rats to ethanol in drinking water. By introducing a completely liquid diet containing ethanol the aversion was overcome. This model was nutritionally adequate and showed for the first time that alcohol may lead to liver damage despite sufficient nutrition. Steatosis, apoptosis and structural alterations in mitochondria and endoplasmatic reticulum are seen. Inflammation and cirrhosis were more severe when the diet was further developed to contain vitamin A, choline and methionine (Leo and Lieber 1983). This model was further developed by Lindros *et al.* by increasing unsaturated fat, lowering carbohydrates, and adding LPS to mimic the human situation of increased circulating endotoxin levels in alcoholics. In animals co-treated with EtOH and LPS, mRNA levels of TNFα and IL-1β were significantly increased (Lindros and Jarvelainen 1998).

1.4.2.2.2 Tsukamoto-French model

The Tsukamoto-French model is based on intragastric feeding of a nutritious diet containing EtOH fortified with vitamins, antioxidants and minerals (Tsukamoto, Towner et al. 1986). The model has been further developed as to result in signs resembling human cirrhosis using carbonyl iron supplementation. Animals develop fatty liver, hepatomegaly, balloon degeneration, apoptosis, necrosis, fibrosis and inflammation (Tsukamoto, Horne et al. 1995). Nanji *et al.* have shown that liver damage in this model is highly dependant on dietary composition, and that saturated fats may prevent damage, while unsaturated fats, particularly those rich in omega-3, aggravate damage (Nanji and French 1988). Others have used the Tsukamoto-French model to show that liver injury is reduced by antibodies towards TNFα.

In this model, animals display a cyclic pattern of fluctuating blood ethanol levels despite constant intragastric infusion. It is speculated that this may be due to cyclic levels of alcohol dehydrogenase (Badger, Ronis et al. 1993). The cycling pattern of urine ethanol concentrations is depicted in the figure below. Both of the models used in this thesis have the base in the Tsukamoto-French model of intragastric feeding.



To the left:
Figure 2. Urine ethanol profiles for ethanol-infused rats given a diet in the style of the Tsukamoto-French model, illustrating the pulsatile pattern of ethanol concentration observed. Each graph represents an individual animal. Modified from Paper III.

2 AIMS

The overall goal for the studies presented in this thesis was to better understand drug and alcohol induced liver toxicity. In essence, this goal was approached from two completely different directions; *in vitro* and *in vivo*. *In vitro* studies aimed at characterizing and trying to further develop the current common *in vitro* models for hepatotoxicity. *In vivo* studies aimed at characterizing and better understanding one of the main liver toxicants of today – alcohol.

More specifically, the *in vitro* studies were aimed at:

- Characterizing the B16A2 human hepatoma cell line to determine its potential for *in vitro* toxicity screening in comparison to one of the most commonly used hepatoma cell lines.
- Further developing the traditional concept of *in vitro* hepatotoxicity models by introducing immunocompetent cells.

The *in vivo* studies were aimed at:

- A broader understanding of the inflammatory effects of alcohol.
- Characterizing inflammation in the absence of CD14 upregulation.
- Further dissecting the role of reactive oxygen species in alcoholic liver disease; by use of antioxidants (N-acetylcysteine) or pro-oxidants (addition of CYP2E1).

3 METHODS

The materials and techniques used in the presented works are more thoroughly described in the manuscripts of this thesis.

3.1 CO-CULTURE MODEL

The aim of the co-culture model was to study interactions between hepatocytes and monocytes, and determine if cells interact so as to increase the sensitivity to toxic insult in the *in vitro* system. Kupffer cells are important in the pathogenesis process of alcoholic liver disease, in cholestatic liver injury (Gehring, Dickson et al. 2006), as well as for hepatotoxicity of metals such as iron or copper (Videla, Fernandez et al. 2003). T cells have been shown to be more important in viral infections (Schumann, Wolf et al. 2000). At the time of the startup of this project, the only Kupffer cell line available was a murine periportal cell line, developed by Dory *et al.* in 2003 (Dory, Echchannaoui et al. 2003). The periportal Kupffer cells are more specialized in phagocytosis and less in cytokine production, which was the focus of our interest. Nowadays more cell lines are available (Peng and Murr 2007). However, an *in vitro* system based on human cells was most desirable. As human material is scarce, and demands of high throughput and reproducibility have to be met, there are not many other options than to use human cell lines. As there was no human immortalized Kupffer cell line at the time, our choice was that of the human monocytic cell line THP-1. These were combined with the human hepatoma cell line Huh-7 for the co-culture system.

We decided to initially set up three different variants of the system. One where cells would come in direct contact, one where cells were separated by a thin layer of collagen as a sandwich culture, and one where cells were separated by a thin porous membrane. We evaluated several parameters in these variants of co-cultures. Our aim was to be able to separately monitor mRNA expression for each cell type. This proved to be very difficult in the systems where cells had direct contact due to contamination, and also in the collagen-separated cultures partly due to the difficulty of extracting RNA from cells in collagen mass. We also made a preliminary experiment to measure the expression of IL-1β and TNFα in the three different proposed models, to get indications of monocyte activation level. Here it was noted that the largest response of pro-inflammatory mediators after troglitazone stimulation (24 hrs, 50μM) was seen in the insert model. Together, these results lead us to choose the insert model, where THP-1 cells grow on a porous permeable membrane (3 μM pore size), 1 mm above a confluent layer of Huh-7 cells. Huh-7 cells seeded at 75 x 10^4 cells/well the day prior to start of the experiment were considered to be confluent. The amount of the non-adherent THP-1 cells was chosen not upon criteria of cell amount corresponding to confluence, but as the lowest amount of cells from which it would be possible to isolate RNA, due to the fact that monocyte content in liver is minor when compared to hepatocyte content. The THP-1 cells were seeded at a density of 30 x 10^4 cells/well the day after Huh-7 cells were plated.

The set up of the co-culture system is illustrated below:

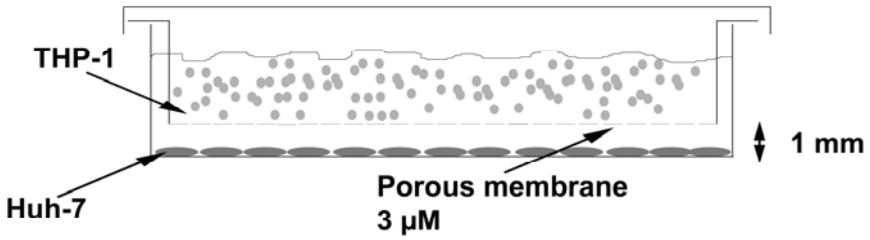


Figure 3. Schematic view of the co-culture system. The THP-1 cells are in the upper compartment of the system, while confluent Huh-7 cells are in the lower. The upper part of the system is separated from the lower part by a porous membrane.

3.2 GENE EXPRESSION ANALYSES

There are many different types of microarrays to study gene expression levels, some may be custom built like spotted arrays. However, we have used two sorts of ready built arrays.

3.2.1 Affymetrix arrays

The Affymetrix gene expression oligonucleotide microarrays are made up of oligonucleotides synthesized *in situ* on a silica microchip. The oligonucleotide arrays are spiked with control genes for normalization. Affymetrix has also developed the software Gene Chip, which has been used for analysis of arrays under standard settings for all Affymetrix related papers in this thesis. Data has then been further processed using analysis of variance (ANOVA) analyses. After normalization and initial ANOVA analyses, certain analyses and visualization of data have been created using the software Spotfire, for instance the hierarchical clustering tables in paper I, and the PCA analyses in paper IV. The Gene Ontology database has been used to provide annotation to datasets. Further annotations have in certain cases been done manually by literature search (PubMed, MGI).

3.2.2 SuperArray

The SuperArray is a PCR based system for specific gene expression analysis in a modified standard 96 well PCR plate. The system is based on SYBR Green detection, and contains a panel of genes of interest together with five housekeeping genes and three RNA quality controls. The SuperArray system is also accompanied by a small computerized protocol for data analysis, which was used with standard settings in paper V. There are numerous different arrays developed by the manufacturer to target different specific needs, we chose to use the Human Stress and Toxicity Pathway

Finder. This array contains 84 different genes of interest in various categories of cellular stress, such as necrosis and apoptosis (ex. annexin V, FasL), oxidative stress (ex. catalase, glutathione reductase), and inflammation (IL-6, iNOS).

3.3 QUANTIFICATION OF MESSENGER RNA

There are several real-time PCR based methods available to quantify mRNA.

3.3.1 SYBR Green and TaqMan

SYBR Green and TaqMan represent two different ways to bind fluorescent molecules to double stranded DNA, and can hence be used for quantification of mRNA using PCR.

SYBR Green real time PCR has been used in paper I, II, III and V. SYBR Green is a fluorescent dye, which exhibits little fluorescence when in solution, but a strong signal upon binding to double-stranded DNA, regardless of the type of double stranded DNA. This is one of the drawbacks of the methods. Due to this characteristic, it is of utmost importance to control for contaminating products when using SYBR Green. These can be visualized using a melt curve, where fluorescence of the final product/products is measured, during continuous increase in temperature. The melting of a specific product creates a sharp drop in fluorescence as the dye no longer binds. Using the derivative of the melting curve, one can control for the presence of contaminants such as more than one product, primer dimers, or primer hairpin loops. This was done for all primer pairs used here to certify specificity of measurements. Also, when primers were set up, the product was run on an agarose gel to control for contaminants.

TaqMan analyses were used in paper IV.
TaqMan instead relies on the use of specific probes with a fluorescent dye bound to the 5´ end and a quencher at the 3´ end. The probe is designed to bind between the flanking primers that produce the PCR product. The proximity of the fluorescent dye to the quencher prevents detection of a fluorescent signal. However, DNA polymerase has 5´-nuclease activity, thus when the polymerase replicates a template to which probe is bound, the 5´end containing fluorescent dye is cleaved off from the probe and will emit a signal. Due to the requirement of 2 primers and 1 probe containing fluorescence and quenchers, TaqMan requires meticulous design which can be time consuming. Hence several companies provide ready designed primer plus probe sets for many genes. Ready designed probes and primers from Applied Biosystems were used in our studies.

3.3.2 Methods for absolute quantification

For absolute quantification used in paper I, standard curves of the final PCR product were made, down to copy numbers of 8 copies per reaction. Calculations were based on the molecular weight of the PCR product. Due to the sensitivity of these dilutions, standard curves were not frozen/thawed more than twice. Nowadays, standard curves of

the PCR product are not commonly used because of the risk of contamination and as the method has difficulties with precision.

3.3.3 Methods for relative quantification

For relative quantification used in paper II, III, IV and V, a random sample was chosen as reference sample, regardless of experimental group. The delta delta Ct method, adjusted for the efficiency of each primer pair was used for the relative mRNA quantifications presented in this thesis. The ddCt method has become more or less standard for relative quantification of mRNAs using real time PCR (Yuan, Wang et al. 2008; Yuan, Reed et al. 2006). To be performed correctly, the method needs to take into account the efficiency of the PCR reaction for each individual primer pair, which can be estimated using standard curves. This was done for all primer pairs used in this thesis.

3.3.4 Selection of housekeeping genes

Several different genes chosen for normalization are present in this thesis. The choice of genes for normalization is highly debated; however, any gene proven not to vary (in relation to total RNA or total cell mass/tissue grams) among different experimental groups in a study can be used. This is the basis for the different normalization-genes chosen in this thesis.

4 RESULTS

4.1 *IN VITRO* STUDIES – PAPER I AND V

The common aim of these works was to refine and further develop the current *in vitro* methods for hepatotoxicity. There is a myriad of human hepatoma cell lines available, derived from various kinds of tumors. Hepatoma cells are essential for the ability to test drug toxicity as primary human material is not available in large amounts, and perhaps not even desirable due to interindividual variability. The human hepatoma cell line HepG2 is undoubtedly one of the most commonly used, (and has been so since the 1980´s), however has the same drawbacks as most other hepatoma cell lines, e.g. a de-differentiated state and lower amounts, or complete lack of, drug metabolizing enzymes. Complementary alternatives to this, or even replacement methods, is most desirable.

4.1.1 Paper I

This work deals with a more in-depth characterization of a hepatoma cell line, developed by Le Jossic and co-workers in 1998 (Le Jossic, Glaise et al. 1996). The reason for a more thorough characterization of the B16A2 cell line was that that it seemed highly differentiated and there were indications that cells were phenotypically becoming more differentiated over longer confluence time. It also expressed the CYP2E1 enzyme, a rare characteristic in human hepatoma cells, as well as inducible CYP1A2. In order to determine the potential of B16A2 as a model for *in vitro* toxicity screening, cells grown at long-term confluence were subjected to whole genome mRNA expression profiling by use of microarrays. B16A2 cells at 0, 2 and 5 weeks of confluence were used. HepG2 cells were assayed for comparison. HepG2 cells were only grown until confluence, as we did not find reports that HepG2 displays similar differentiation characteristics over time. Also, a pool of in total 9 human livers was used as a reference.

Analyses showed that the majority of genes expressed were common between the two cell lines and adult human liver. We also showed that many different groups of genes were affected by confluence-time in the B16A2 cell line. More changes were observed between week 2 to 5, than between week 0 and 2. Several classes of genes known to be important for metabolism, particularly lipid and protein metabolism, as well as drug metabolism showed changes in gene expression pattern. In general, two trends were noticeable that in the end made the 5 week B16A2 cells resemble human liver more than the 0 week cells. Genes with high expression in the human liver were increasing in expression over time of confluent growth in B16A2 cells. Also, genes very high expression in the hepatoma cells, sometimes known to be associated with tumors and a de-differentiated fetal stage, decreased in expression. We also searched for expression of mRNAs that could be thought of as liver specific “markers”, such as albumin that significantly increased its expression about 33-fold. CYPs such as CYP3A4 and CYP2E1 significantly increased their mRNA expression, about 7-8 fold. However,

when CYP induction was studied in relation to rifampicine (e.g. induces 3A´s) and phenobarbital (induces CYP2B´s) treatment of B16A2 cells, no significant induction was found. This would indicate that cells may be lacking transcription factors relevant for achieving functional induction.

This was one of the reasons why we chose to determine the levels of liver enriched transcription factors in all the cells/livers studied. A small selection of different transcription factors was analyzed; HNF-1α, -4α, -6, Oct-1 and DBP. The results of these analyses showed that both cell lines contained mRNA amounts in the same scale of magnitude as adult human liver (within about 3-fold differences), while HNF-4α was even higher expressed in the HepG2 cells than human liver. We also looked at PXR and CAR, both of which were found not to be expressed in B16A2 cells.

4.1.2 Paper V

The general idea behind this paper is to test if current standard *in vitro* toxicity testing may be improved by introducing another cell type into the system. The main parenchymal mass of the liver is as mentioned that of hepatocytes, accompanied by 3 other major cell types; the liver endothelial cells, stellate cells and Kupffer cells. The main inflammatory cells of the liver are Kupffer cells, NK and NK T cells. Due to the choice of developing a human system, cell lines were used instead of primary cells.

Several different conditions were tried for the chosen monocyte and hepatoma cell lines to coexist, with the demand that they should be possible to separate and analyze, as mentioned in the methods section. A number of different analyses led us to choose the model similar to the one developed by Cederbaum *et al*. (Nieto and Cederbaum 2003).

The aim was to see if a co-culture could predict toxicity with a higher sensitivity than either of the cell types in single culture. We decided to test a pair of hepatotoxic/non-hepatotoxic drugs on this system for an initial evaluation. Troglitazone and rosiglitazone are both PPARγ-agonists and have very similar structures. However, troglitazone has been proven to be liver toxic, while rosiglitazone at the start-up of our studies and until recently, was considered a non-toxic member of the PPARγ agonist family. To our knowledge, there has been only one case of rosiglitazone hepatotoxicity reported (Al-Salman, Arjomand et al. 2000).

A preliminary screening of gene expression in troglitazone treated cells in co-culture at different time-points was used to find a selection of stress related genes, which were studied more in depth and compared between tro- and rosiglitazone. This was done using the PCR based array from SuperArray described in the methods section. SYBR Green real time PCR methods were then set up for these genes. We found that troglitazone induces expression of several stress related genes such as DDIT3/CHOP, MT2A, CXCL2, CXCL10, and HspA6 in Huh-7 cells in co-cultures as compared to single cultures. All mRNAs mentioned above except for HspA6 also showed increased expression in THP-1 cells in co-culture as compared to separate single cultures. Troglitazone treated cells were not only more sensitive in co-culture, but also expressed higher levels than rosiglitazone treated cells.

We analyzed the viability of cells in the system, and concluded that viability after troglitazone treatment decreases 30-40% for both cell types when grown in co-culture in comparison to the troglitazone treatment given to single cultures. THP-1 cells showed a substantially decreased viability at an earlier time-point in co-cultures than in single cultures (8 hrs instead of 24/48 hrs). For Huh-7 cells, extensive death was seen at 48 hrs in single-cultures, but at 24 hrs in co-cultures.

4.2 *IN VIVO* STUDIES – PAPER II, III, AND IV

The common aim of these three studies was to get a deeper knowledge about the development of alcoholic liver disease. Today there are several rat models available, like the previously mentioned Lieber and DeCarli-model (DeCarli and Lieber 1967), and the Tsukamoto-French model (Tsukamoto, Towner et al. 1986). Despite various different models for alcoholic liver disease, there is still debate whether any of these are fully sufficient (de la, Lieber et al. 2001). An option to somewhat try to compensate for the lack of one particular model, is to combine knowledge from several different methods for a broader understanding.

4.2.1 Paper III

The goal of this study was to see the expression of Th1 and Th2 type cytokines during the early development of alcoholic liver disease, using a low carbohydrate rat model developed by Badger and Ronis *et al*. (Badger, Ronis et al. 1993; Korourian, Hakkak et al. 1999; Ronis, Hakkak et al. 2004).

Animals were given ethanol via total enteral nutrition and followed for up to 49 days. Urine ethanol concentrations were measured daily. The urine ethanol concentration follows a pulsatile pattern which peaks at intervals of about 6 days (Paper 2, Fig.1, Paper III, Fig. 1). Rats were sacrificed at peaks and nadirs of these pulses. Ethanol treatment resulted in significant steatosis and lipid peroxidation. Inflammatory infiltrates, as well as local foci of necrosis and apoptosis were also seen. CYP2E1 was highly induced which was shown on a protein level and by para-nitrophenol oxidation, additionally induction was also significant on mRNA level.

Th1 type cytokines like TNFα showed a significant upregulation at day 14, then decreased back to control levels, after which a second peak occurred at day 35. This biphasic pattern was mirrored by the TNFα protein expression levels. INFγ showed a similar pattern, with mRNA increasing significantly at day 14, also accompanied by similar changes in protein levels. However, IL-12, driving Th1 T cell differentiation, showed suppressed expression from day 21 until 42-49. The lowering of IL-12 was accompanied by decreased expression of the Th1 regulating transcription factors T-bet and STAT-4 on a protein level.

Th2 type cytokines were generally harder to detect. IL-5 and IL-13 mRNAs were below detection levels. However, IL-4 mRNA was clearly detectable and showed a

very drastic decrease in expression throughout the entire study. This could however not be confirmed on a protein level as IL-4 protein levels were below detection limit. This was accompanied on a protein level by decreases of the Th2 associated transcription factor GATA-3.

Cytokines were also studied in relation to pulsatile blood alcohol levels. TGFβ increased with increased blood ethanol, while IL-12 showed the opposite pattern.
The chemokine CXCL2/IL-8 did not differ significantly in expression between peaks and nadirs of EtOH concentrations, but increased continuously over the study period, only to decrease the last week. CINC1/GRO also showed a similar pattern of continuous increase, but in contrast to IL-8 it differed significantly between the peaks and nadirs.

Hepatocyte proliferation was assessed on animals sacrificed at days 14 and days 42-49, the same time points that showed peaks in TNFα expression. It was shown that there was an increase of cells in G1, S, G2 and M phases at the latter time point (paper III, Fig. 9).

4.2.2 Paper II

The presence of lipid peroxidation products is only one of several factors indicating that oxidative stress is a major factor in the pathogenesis of alcoholic liver disease. Also, it has been postulated that oxidative stress could influence the inflammatory status of the liver from several aspects, such as activation of monocytes and production of antibodies (Jayatilleke and Shaw 1998; Mottaran, Stewart et al. 2002). Also, from the previously mentioned paper III, and others it is clear that cytokines play a major role in the development of alcoholic liver disease (Yin, Wheeler et al. 1999). We decided to focus on the effects of antioxidants on progression of alcoholic liver disease. The same model as in paper III was used and antioxidants studied were butylated hydroxytoluene and N-acetylcysteine (NAC). However, significant effects were not seen with butylated hydroxytoluene, and hence the paper was focused on NAC effects.

As in the previous study, EtOH resulted in steatosis, inflammation, focal necrosis and alanine aminotransferase elevation. CYP2E1 was again highly induced. Also here we could see an increased TNFα expression, and a decreased expression of IL-4. Lipid peroxidation products were also measured, and protein adducts in the form of MDA, HNE and hydroxyethyl (HER) were formed. Rats had an ongoing immune response as shown by antibodies towards HNE and HER.

NAC was shown to significantly affect many of these parameters without affecting urine ethanol concentrations. ALT increase after EtOH administration was significantly diminished, but not abolished, in the EtOH treated animals given NAC in comparison to animals only receiving ethanol. The Oxygen Radical Absorbance Capacity assay was used to determine the levels of cytosolic antioxidants in livers, and animals treated with NAC had higher basal levels of antioxidant capacity. Antioxidant capacity was decreased with approximately the same amount in EtOH and EtOH + NAC animals. But as the NAC animals had a higher basal level, the levels in EtOH + NAC animals

were similar to untreated control animals. GSH concentration was also measured in liver homogenates as another way to study redox status. By this it was shown that EtOH decreased liver GSH, but this was prevented by NAC treatment. Immunohistochemical methods were used to look for lipid peroxidation protein adducts. Also here, NAC could more or less abolish protein adducts (MDA and HNE adducts). Antibodies against these protein adducts have been shown to play a part in alcoholic liver disease (Albano, Mottaran et al. 2005). Additionally we studied antibodies directed against neo-antigens formed by MDA, HER and HNE protein adducts. These were all significantly higher in EtOH treated animals. By treatment with NAC all these antibodies decreased, of which HNE showed a drastic significant decrease. Steatosis-score was not affected by NAC. Neither was induction of CYP2E1.

A multitude of cytokines were measured in this study – TNFα, TGFβ, IL-1β, IL-4, and IL-6. NAC treatment produced a ca 50% decrease in basal IL-6 expression. IL-4 was lowered in the EtOH-treated animals, similar to what was shown in paper III. Also here, TNFα and TGFβ increased significantly in EtOH treated animals. TFGβ, IL-4 and IL-6 mRNA expression were not affected by NAC treatment when EtOH was present. TNFα however, showed a mean decrease of expression of about 40%. However, due to large interindividual variation between animals, the numbers did not reach statistical significance. TNFα levels also showed significant correlation to pathological score, and MDA and HRE protein adducts.

4.2.3 Paper IV

This paper uses a different model than the other two *in vivo* papers. Mice in general have very low levels of CYP2E1 and it is harder to induce liver damage in mice than in rats, hence rats are used in several models of ALD. As rats develop more liver damage an ideal model to study the effect of CYP2E1 on ALD would be a transgenic rat model. However, until recently it was not possible to develop transgenic rats. Hence our choice was to use a transgenic mouse, but still a diet in the style of Tsukamoto and French's as previously mentioned (Tsukamoto, Towner et al. 1986). This mouse model was developed by the group of Morgan *et al*. in 2002, and is transgenic for the human CYP2E1 enzyme (Morgan, French et al. 2002).

CYP2E1 is normally highly induced in alcoholic liver disease, and it has been proposed that oxidant species leaking out from this enzyme during metabolism of substrate damages the liver (Albano, French et al. 1999). This study was conducted to further shed some light into the role of oxidant species in liver disease, and complements the antioxidant treatment in paper II.

Similarly to the two previously described papers, measurements of ALT, lipid peroxidation products (MDA) and para-nitrophenol activity were performed. Para-nitrophenol oxidation is a specific marker of CYP2E1 activity in mice (Wolf, Wood et al. 2004). Histological examination determined scores for inflammation, total fat (divided into micro- and macro-vesicular fat), and necrosis. An initial PCA analysis was done on the analyses/measurements mentioned, showing that the largest component of variation (PC1) correlated directly to pathology score, and was highly

influenced by para-nitrophenol activity. This showed a connection between total pathology score and para-nitrophenol activity.

Mice were also subject to global gene expression analysis using Affymetrix microarrays. It was noted that the number of transcripts determined as P, Present, by the Affymetrix software was about equal in all groups of mice (+/- alcohol, +/- transgenic for 2E1). There were hardly any genes that were changed in animals given dextrose, when comparing the non-transgenic to the transgenic group. However, there were large differences in gene expression when comparing dextrose treated animals to EtOH treated, and this was common for both the transgenic and non-transgenic animals. Genes changing in expression by EtOH in both animal groups were classified by Gene Ontology, and the largest groups of genes up regulated by EtOH were glutathione transferases and monooxidases. Genes decreasing in expression by EtOH were not as well characterized and consisted mostly of mRNAs of unclassified function. The comparison of transgenic and non-transgenic animals given EtOH targets the most interesting question – what effect CYP2E1 has on gene expression in a context where high amounts of the enzyme is needed. It was shown that approx. 30 genes were up regulated in the transgenic + EtOH animals, in comparison to non-transgenic EtOH. Among these were TGFβ1 induced transcript 4 and fatty acid desaturase 2. Decreased mRNA levels were seen for hemoglobin α and β chains, and the Ah-receptor.

A list of genes correlated to pathology score was also made, by correlation analyses using Pearson correlation coefficient. Among the top genes correlated to pathology were several genes in the keratin complex, like cytokeratin 8 (keratin complex 2, basic gene 8).

Genes for confirmatory analyses by TaqMan real time PCR were chosen from the gene-lists. Both genes up- and down- regulated by EtOH were chosen. Also, some genes were chosen due to their correlation to total pathology score. In general, these analyses could confirm what was seen in the microarray experiments. However, variation was seen: some genes showed larger differences between groups when studies by real time, while some differences were not significant.

5 DISCUSSION

5.1 *IN VITRO* MODELS FOR HEPATOTOXICITY

Drug-induced hepatotoxicity is one of the most common causes of drug withdrawal at the later stages in drug development (Phase II and onwards) (Kaplowitz 2005). As patients are receiving the drug, and a lot of resources have already been put into compound development, the need for better prediction of hepatotoxicity at an earlier stage cannot be overemphasized.

It should also be noted that primary hepatic cells are not an optimal model either, as hepatocytes lose their ability to express drug metabolizing enzymes very rapidly after being plated. In the first phases of pre-clinical drug development, hepatotoxicity has traditionally been performed on hepatocytes, either primary from rodents, or human cell lines.

5.1.1.1 Chemical inducers of differentiation

In many studies, different chemical agents such as DMSO are used to induce or speed up differentiation (Gripon, Rumin et al. 2002; Plescia, Rogler et al. 2001). In the years following the publication of this study, the development of more adult-like human hepatocytes has been more focused on stem cells, also here there exists a myriad of different chemical protocols to induce differentiation and maturation (Levenberg, Huang et al. 2003). DMSO has not only been used for differentiation of a variety of adult cell types but also in stem cells (Hallows and Frank 1992; Pratt, Crippen et al. 1998; Lako, Lindsay et al. 2001). Activin A has been particularly used for differentiating stem cells into hepatocyte-like cells (Soto-Gutierrez, Navarro-Alvarez et al. 2007). Some of these stem-cell derived cells have been shown to be able to express drug metabolizing enzymes (Ek, Soderdahl et al. 2007). Despite the recent trend aiming at differentiating embryonic stem cells into cells of an adult hepatic phenotype (Asahina, Teramoto et al. 2006; Kumashiro, Asahina et al. 2005; Banas, Yamamoto et al. 2007) a functional adult hepatic phenotype will probably take many more years to be developed. As primary hepatic cells lose many of their functions rapidly, even if adult-like cells would be possible to develop, the challenge would to maintain hepatic functions over an extended period of time will require further efforts. This development is probably rather distant, and until then primary hepatocytes and hepatoma cell lines remain a decent alternative.

Unlike the examples mentioned above, in paper I in this thesis, chemicals were not given to drive differentiation; hence the results can be seen more as changes in gene expression in relation to cell-cell or cell-matrix interaction. Fig.1, paper I show that changes in mRNA expression over time in B16A2 makes the expression pattern of the cell line more similar to adult human liver. Many genes determined to have a lower expression in human hepatocellular carcinoma increased their expression over confluence time in B16A2 (Xu, Huang et al. 2001). However, as changes are more

drastic between week 2 and 5 than between 0 to 2, this indicates that long term confluent cells are needed, which is a technically troublesome procedure on a large scale. And when looking at absolute levels of expression, many genes, although up regulated, did not reach levels which would be comparable to human liver. Liver enriched transcription factors were in several cases higher in HepG2 than in B16A2, and this cell line also had a more adult-like expression pattern for Phase II enzymes.

5.1.1.2 Novel hepatoma cell lines

Other attempts to develop better hepatoma cell lines are the HepaRG cells that at confluence form hepatocyte-like structures, surrounded by biliary epithelial-like cells. In contrast to HepG2 cells and other hepatoma cell lines, HepaRG cells express a variety of P450´s, like CYP1A2, 2B6, 2C9, 2E1 and 3A4. They also express the two intracellular receptors that were lacking in B16A2 cells as studied in paper I, constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Guillouzo, Corlu et al. 2007). Drug metabolizing enzymes have indeed been shown to be inducible in the HepaRG cells. Treatment of cells with known inducers resulted in increased expression of all the CYPs previously mentioned as well as CYP1A1, CYP2C8, and CYP2C19 mRNA (Kanebratt and Andersson 2008), features which are unusual for hepatoma cells.

5.2 INFLAMMATORY CELLS IN HEPATOTOXICITY

It is clear that monocytes and macrophages play important roles in liver injury. The liver resident Kupffer cells are the main mass of tissue resident macrophages (Czaja, Geerts et al. 1994). Considering the importance of these cells for liver injury, and the insufficiency of methods consisting of one cell type only, we introduced the human monocytic cell line THP-1 into a co-culture system together with the hepatoma cell line Huh-7. This system showed a lower viability in the co-cultures when stressed with a known hepatotoxin, troglitazone (fig 2, paper V).

5.2.1.1 Transcriptional changes in troglitazone co-cultures in relation to mitochondrial stress

Troglitazone is known to produce mitochondrial damage at high levels (Ong, Latchoumycandane et al. 2007). The mRNAs for DDIT3/CHOP, MT2A and CXCL10 and CXCL2 were significantly higher in the co-cultures as compared to single-cultures (Fig. 5 and 6, paper V). Transcription of DDIT3 is regulated by mitochondrial oxidative stress (Carriere, Carmona et al. 2004). The increase in DDIT3 in our studies is in line with others who show that troglitazone leads to increased mitochondrial stress, like increased mitochondrial membrane permeability as reviewed for instance by Jaeschke (Jaeschke 2007). In cell lines like HepG2 it was shown that troglitazone decreases mitochondrial membrane potential (Bova, Tam et al. 2005), and a mitochondrial permeability transition inhibitor could completely protect against troglitazone-induced

cell death (Tirmenstein, Hu et al. 2002). Also *in vivo* increased mitochondrial stress has been shown in rats with an otherwise silent deficiency in mitochondrial function, this occurs partly by way of decreased activity in complex I (Ong, Latchoumycandane et al. 2007). This may indicate that troglitazone has the potential to lead to mitochondrial damage, and that some otherwise healthy individuals are more susceptible. MT2A expression has also been shown to be up regulated by increased oxidative stress in relation to deficiencies in mitochondrial complex I *in vitro* (Reinecke, Levanets et al. 2006). Interestingly, in our experiments both these effects on mitochondrial function were significantly more pronounced in co-cultures than in single cultures. This data would point to that oxidative stress, perhaps particularly mitochondrial oxidative stress, plays a role in troglitazone toxicity. However, out of 3 antioxidants tried here: NAC, vitamin E derived trolox, and catalase, only catalase showed a significant effect. We had an effect of NAC *in vivo* after EtOH treatment in paper II, where it was shown that cytokines like TNFα tended to decrease in expression after NAC treatment of animals given EtOH. Whereas in the co-culture system, we only see a difference with catalase. It has been argued that particularly when it comes to mitochondrial damage, traditional antioxidants may not suffice (Bai and Cederbaum 2001). It has also been shown that both cytosolic and a particular form of mitochondrial catalase can protect HepG2 cells from apoptosis (Bai and Cederbaum 2001; Bai, Rodriguez et al. 1999). Perhaps this is the reason why we achieve effect only with catalase.

5.2.1.2 Cytokines and chemokines in the in vitro co-culture system

The chemokine CXCL2/MIP2, has a role in recruiting neutrophils to sites of damage. CXCL2 expression is regulated by NF-κB, which in turn is activated by oxidative stress for instance after toxic insult (Jokelainen, Reinke et al. 2001), (Driscoll 2000). Also CXLC10 is a main chemokine to drive neutrophil recruitment, and is also activated by oxidative stress (Michalec, Choudhury et al. 2002). Cytokine expression was not increased dramatically between the single- and co-culture systems. TNFα, probably the main pro-inflammatory cytokine, showed similar expression in single- and co-cultures. Our data indicates that lower viability in co-cultures is not related to a classic inflammatory Th1 response, but probably more a reaction to increased oxidative stress.

In summary, we could see an increased cell death in co-cultures in comparison to single cultures for the toxic troglitazone but not for the non-toxic rosiglitazone. This, rather than deciphering the exact mechanism of death, is what could potentially make the system useful for drug toxicity screening. Our future plans include continuing to stress the cells with other hepatotoxins and determine if increased sensitivity is seen in the co-culture system.

5.3 ALCOHOL HEPATOTOXICITY

5.3.1 Inflammatory response and liver damage

In paper III we show that a careful monitoring of urine EtOH revealed EtOH levels to be pulsatile, despite chronic constant EtOH infusion. This is in accordance with previous studies (Badger, Hoog et al. 2000; Badger, Ronis et al. 1995), and provides a unique opportunity to see if inflammatory me diators can be affected by the absolute levels of EtOH in the body, not only by the duration of treatment.

5.3.1.1 Th1 response in animal models for ALD

IL-12 mRNA was significantly decreased at time points when EtOH concentrations were highest (paper III, fig. 7). It is known that EtOH suppresses IL-12 expression *in vivo* (Pruett, Fan et al. 2005). Paradoxically, IL-12 may also be increased by EtOH, particularly as an acute effect (Szabo, Girouard et al. 1996). Also in our study, at the first studied time-point we see an increase in IL-12 production (paper III, fig. 4). After some time, IL-12 decreases. To explain this complex pattern, of both up- and down-regulation, is difficult. However, it has also been noted that IFNγ levels are critical for determining EtOH effects on IL-12 (Girouard, Mandrekar et al. 1998). Additionally, the regulation of IL-12 secretion depends on many different factors: LPS levels, IL-10 levels (Girouard, Mandrekar et al. 1998), and intracellular cAMP (Szabo, Girouard et al. 1998). These factors were not all studied in our paper, and may have been changing over study time.

TNFα showed a trend, however not significant, to be increased when EtOH concentrations were highest (paper III, fig7). This is in line with findings of Kishore *et al.* showing stabilization of TNFα mRNA by EtOH (Kishore, McMullen et al. 2001). TNFα is known to be of major importance in ALD development (Yin, Wheeler et al. 1999; Crews, Bechara et al. 2006), and is significantly up regulated after EtOH treatment in our studies despite a lack of endotoxemia. Our results show an initial upregulation of Th1 response (TNFα, IFNγ, IL-12) early in the time course (paper III, fig. 4), followed by a fluctuating pattern decreasing and then increasing again. These are known to be the "initiating" cytokines.

5.3.1.2 Th1 versus Th2 balance, fibrosis and regeneration

It has been suggested that an initial inflammatory Th1 response may be followed by a long-term fibrinogenic Th2 response (Crews, Bechara et al. 2006). In general this was not seen here, as IL-4 was suppressed throughout the entire study (paper III, fig.4), and fibrotic lesions were not shown. However, we did see a significant increase in TGFβ mRNA expression at the time of maximal blood EtOH levels (paper III, Fig. 7). As TGFβ has been shown to be of large importance for induction of collagen synthesis in stellate cells, this may be seen as an early indicator of fibrinogenic response (Friedman and Arthur 1989; Friedman 2008). As fibrosis is a later stage in disease development, perhaps it would require a longer duration of EtOH treatment than used here. Apart

from this our data are generally more in line with an acute innate immune response to EtOH, as proposed by Wheeler and colleagues (Crews, Bechara et al. 2006). Transcription factors governing Th1 and Th2 response were also studied. A reduction in GATA3 was shown, consistent with lowering of IL-4 expression. Reductions were also seen of T-bet and STAT-4 (paper III, Fig. 6), consistent with the decrease in IL-12 following the initial peak.

It has been noted that TNFα may be related to liver regeneration (Baumgardner, Shankar et al. 2007; Isayama, Froh et al. 2004). In our study, an increase in hepatocyte proliferation was seen at day 42-49 (paper III, fig 9), corresponding to the second peak of TNFα expression. Exactly what cell type is producing these effects however remains to be studied. As mentioned, the liver has one of the body's largest resident macrophage populations, as well as a large amount of NK T cells. Other types of T cells as well as peripheral blood monocytes (PBMC) may also migrate to the liver (Laso, Vaquero et al. 2007). CXCL2, the rat analogue of IL-8, was shown to have a continuously increasing expression during long-term EtOH administration (paper III, fig8). This may contribute to inflammatory infiltrates and PBMC recruitment, which could then contribute to the second TNFα peak.

5.3.2 Oxidative stress in relation to liver damage

In both paper II and III, animals developed steatosis, inflammation and necrosis after several weeks of EtOH treatment. ALT levels were also significantly increased. We show in paper II, fig. 3 that NAC could provide significant protection against this ALT elevation. However NAC does not protect against steatosis, which is often seen before severe pathology develops.

5.3.2.1 Lipid peroxidation products

Lipid peroxidation products have also been implicated in pathogenesis of ALD. It has been shown in alcoholics that lipid peroxidation products can increase collagen production in hepatic stellate cells. This has been shown for HNE (Parola, Pinzani et al. 1996), and MDA to a lesser extent (Maher, Tzagarakis et al. 1994). NAC supplementation increases antioxidant capacity in rats (paper II, fig 3), and also lowers lipid peroxidation products significantly. HNE adducts are almost abolished in EtOH + NAC treated animals. Lipid peroxidation products can form protein adducts that interfere with normal protein function, contribute to formation of Mallory bodies (Letteron, Fromenty et al. 1996), and may interfere with signaling pathways (Sampey, Korourian et al. 2003).

Lipid peroxidation products have an effect on inflammation. It has been shown in human alcoholics that antibodies directed towards lipid peroxidation protein adducts are readily detected and T cells of alcoholics respond to these (Stewart, Vidali et al. 2004). We show here that antibody-titers towards lipid peroxidation products are lowered in EtOH + NAC fed animals, as compared to EtOH fed animals. Antibodies towards all 3 different types of adducts, MDA, HNE, and HER, are significantly

lowered. In the case of MDA, levels after EtOH + NAC treatment are comparable to those of control animals. Considering that lipid peroxidation products are significantly lowered by NAC, it would be expected for NAC to hamper disease progression in ALD. Indeed, we can show a significantly lower pathology score in animals fed EtOH + NAC in comparison to EtOH only. Also, a lowering of necrosis is seen, as well as a significant lowering of ALT (paper II, Table 2).

Several cytokines were also studied in paper II, and it was shown that NAC could drastically reduce TNFα expression in EtOH + NAC treated animals in comparison to EtOH treated. There was also good correlation between oxidative stress markers, inflammation, necrosis and TNFα. The role of TNFα as one of the main culprits in ALD is hardly disputed (Zhou, Wang et al. 2003; Yin, Wheeler et al. 1999).

Hence we show that an effective antioxidant can reverse some of the known effects of lipid peroxidation products, decrease inflammatory factors known to be crucial for ALD development, and provide partial protection to EtOH induced liver injury in animals.

The food preservative butylated hydroxytoluene is used to prevent lipid peroxidation in foods, by acting as a synthetic analogue of vitamin E. It has been shown that butylated hydroxytoluene can decrease NF-κB and reduce EtOH induced brain damage (Crews, Nixon et al. 2006). We also tested butylated hydroxytoluene in the same animal model as was used for the NAC-study. However, we did not see the same significant protective effects of butylated hydroxytoluene in our studies (data not shown). We do not know clearly what lies behind the lack of effect. However, butylated hydroxytoluene is lipophilic while NAC is hydrophilic. Perhaps differences in chemical properties can influence absorption and distribution and hence effect of the compounds.

5.3.3 The potential role for CYP2E1 in alcoholic liver disease

The opposite effect of adding antioxidants would be to add a pro-oxidant. Addition of the human monooxygenase CYP2E1 gene to a mouse model for ALD is studied in paper IV. CYP2E1 has long been suggested to play a role in the development of ALD. Several different authors conclude that it has the possibility to generate superoxide, hydroxyl and hydroxyethyl radicals (Ingelman-Sundberg and Johansson 1984; Ekstrom and Ingelman-Sundberg 1989; Day and James 1998; Misra, Bradford et al. 1992; Gergel, Misik et al. 1997). It has also been implicated as one of the key players in generating free radicals that would then lead to lipid peroxidation (Albano, Clot et al. 1996). The group of Cederbaum has developed an *in vitro* model to study the effects of CYP2E1, where the enzyme is over expressed either by retroviral insertion or transient transfection. Here it is shown that apoptosis occurs to a larger extent in CYP2E1-containing cells, and that lowering of cellular GSH levels leads to an increased cell death in the CYP2E1 containing cells (Cederbaum, Wu et al. 2001). Cell death decreased when antioxidants such as catalase were added to the system (Mari, Bai et al. 2002; Bai and Cederbaum 2003).

5.3.3.1 Different in vivo models to study the influence of CYP2E1 on ALD

There have also been several approaches to asses the potential toxicity of CYP2E1 *in vivo*. Bai and Cederbaum have studied transient adenoviral expression of CYP2E1 in a mouse model where addition of CYP2E1 is shown to elevate the amount of apoptotic cells in the liver (Bai and Cederbaum 2006). There is also a humanized mouse developed by the group of Gonzales and others that has been shown to metabolize CYP2E1 substrates (Cheung, Yu et al. 2005). This humanized model is a CYP2e1 knock-out mouse into which the human CYP2E1 has been inserted. There is also the transgenic mouse containing the human CYP2E1 developed by Morgan *et al*., which we have used in our studies (Morgan, French et al. 2002). Yet another system comprises of a CYP2e1 knockout mouse, which was used to study paracetamol as well as EtOH toxicity. (Kono, Bradford et al. 1999). In the paper by Kono mentioned above, one is studying the removal of relatively low amounts of CYP2E1 to begin with, and compare to a null state. Therefore, models like the adenoviral or the transgenic mouse models mentioned, where additional CYP2E1 is added, is preferred.

As mentioned, we used the model developed by Morgan et al. (Morgan, French et al. 2002) to gain a better understanding of what happens on a whole genome level when mice are under the extra oxidative stress generated by CYP2E1. An initial PCA analysis of the different pathology and clinical chemistry measurements performed (paper IV, fig. 2) showed that the largest component of variation in the dataset was related to treatment group (transgenic/non-transgenic, EtOH/dextrose), which was highly correlated to total pathology. The transgenic EtOH treated animals had the highest total pathology score. Secondly, total pathology was shown to be highly influenced by para-nitrophenol activity mirroring CYP2E1 activity. This indicates that para-nitrophenol activity does have a large effect on pathology development, which was also seen on an mRNA level for several genes known to be important for ALD development.

5.3.3.2 Oxidative stress and protective responses in the CYP2E1 transgenic mouse

We have showed that glutathione S-transferases (GST) were the largest group of genes up regulated by EtOH + CYP2E1 in our study conditions. This is in line with the work of Cederbaum *et al*, that show an upregulation of GSTs in an *in vitro* model of CYP2E1 transfected HepG2 cells (Mari and Cederbaum 2001). It has previously been known that antioxidant genes such as GSTs are up regulated by EtOH treatment only (Vanhaecke, Lindros et al. 2000), but we show that additional CYP2E1 further increases this induction. This might indicate that GST expression is regulated in part by the extra oxidative stress imposed by the CYP2E1 enzyme. Indeed, it has been shown that GSTs are regulated in part by the transcription factor Nrf2 (Ishii, Itoh et al. 2000), that is implicated in oxidative stress response and protective reactions (Cho, Reddy et al. 2006), (Pi, Zhang et al. 2008). Nrf2 can be induced in the previously mentioned *in vitro* CYP2E1 over expressing HepG2 cells (Gong and Cederbaum 2006). The importance of not only GST but also cellular glutathione levels as protective factors towards liver injury, in particular in relation to CYP2E1 has been extensively studied (Cederbaum, Wu et al. 2001; Gong and Cederbaum 2006).

Oxidative stress has been shown to be an important factor in stellate cell activation, and may lead to fibrosis. Also TGFβ is an important activator of fibrinogenic response, as previously mentioned (Friedman and Arthur 1989; Friedman 2008). We showed a significant upregulation of TGFβ1 induced transcript 4 in paper IV, in transgenic EtOH treated animals in comparison to non-transgenic EtOH fed animals.

5.3.3.3 TNFα in relation to oxidative stress and CYP2E1 levels

In paper II, table 3, we show that the addition of an antioxidant to a rat model for alcoholic liver disease decreased TNFα expression, hence addition of a pro-oxidant would be expected to increase TNFα production. This is also what we see with the significant upregulation of TNFα induced proteins 1 and 2, and the confirmatory analyses of TNFα mRNA in paper IV, fig 4. TNFα is known to be one of the main inflammatory cytokines involved in ALD development, as previously mentioned (Yin, Wheeler et al. 1999). TNFα expression has however, to our knowledge, not been studied *in vivo* in relation to CYP2E1 previously. TNFα expression is known to be regulated by oxidative stress in macrophages (Ahmed, Aronson et al. 2000). For future studies it would therefore be of great interest to isolate Kupffer cells from ethanol-treated CYP2E1-transgenic animals, and see if release of TNFα in response to oxidative stress and/or LPS is higher than that of non-transgenic animals.

5.3.3.4 Cytokeratins and formation of Mallory bodies

We also show that several cytokeratins are highly correlated to total pathology score in the animal model in paper IV, as determined by Pearson correlation. Cytokeratin 8 and 18 are the main keratin components of Mallory bodies (Nakamichi, Toivola et al. 2005; Gonzalez-Quintela, Garcia et al. 2006). Cytokeratin 8 ov erexpression has previously been shown to induce Mallory body formation in mice (Nakamichi, Toivola et al. 2005). Cytokeratins 8 and 18 have also been shown to be up regulated in response to oxidative stress by CYP2E1 *in vitro* (Bardag-Gorce, French et al. 2006). The authors propose the following mechanism for the way which CYP2E1 may influence Mallory body formation: increased antioxidants may interfere with proteasomal activity, this will lead to less efficient protein turnover which leads to the accumulation of cytokeratin. Cytokeratins normally have a high rate of turnover (Strnad, Windoffer et al. 2002), but accumulation of these proteins lead to their aggregation into Mallory bodies. Mallory bodies then compromise the function of the microtubule network as well as the centrosomes which will eventually lead to cell death (Nakamichi, Hatakeyama et al. 2002).

In summary, the addition of human CYP2E1 to mice given EtOH has shown to have an effect on pathology. Additionally, searching for genes correlated to pathology showed a high correlation between pathology and genes known to be involved in the formation of Mallory bodies, which are detrimental to cellular function. Also, we show a significant increase of TNFα induced mRNAs in transgenic animals given EtOH, in relation to non-transgenic animals. This would indicate that TNFα expression, already known to

be highly increased by EtOH itself, can to some extent further increase due to the extra cellular stress that elevated CYP2E1 levels provide.

The development of ALD is likely a multifactorial process. Oxidative stress however, plays a major role by increasing lipid peroxidation, and activating macrophages to secrete pro-inflammatory cytokines like TNFα. We could show that TNFα expression can be modulated by varying the levels of oxidative stress in two different *in vivo* systems. Decreasing oxidative stress by addition of NAC to EtOH treated animals results in lower TNFα expression, while additional oxidative stress due to increased CYP2E1 expression increases TNFα expression. Liver pathology was shown to be affected in the same way as TNFα expression by the various means (addition of NAC or CYP2E1) of affecting levels of oxidative stress.

6 CONCLUSIONS

The results of the current thesis can be summarized as follows:

6.1 *IN VITRO* HEPATOTOXICITY

Our studies of human *in vitro* models for hepatotoxicity would indicate that:

- Growing the human hepatoma cell line B16A2 under long-term confluence leads to spontaneous changes in gene expression, which are in most part directed towards a more mature hepatocyte phenotype. Several transcripts coding for proteins with important liver functions are induced – although still very low.

- Both HepG2 and B16A2 hepatoma cell lines express transcripts for several of the most important liver enriched transcription factors in levels comparable to that of adult human liver, *i. e.* within 3-fold difference for all transcription factors studied.

- Co-cultures using a hepatoma cell line and immunocompetent cells in the form of monocytes, as compared to hepatoma cells alone, can give higher sensitivity to a known hepatotoxin like troglitazone.

- Expression of several stress related genes increase in co-cultures during troglitazone treatment, as compared to single cultures. Several of these genes have previously been shown to be regulated by, or to be a response to, oxidative stress, particularly mitochondrial oxidative stress.

In summary, our studies have focused on the challenging need for better *in vitro* models for toxicity testing. Our results shed some light over the potential for new human hepatoma cell lines in comparison to the one most frequently used, and that co-culture systems may be beneficial. Systems like the ones studied here can be a basis for future studies regarding interactions between hepatic and inflammatory cells, and perhaps also for introducing other cell types. It may be that such interactions better mimic the *in vivo* situation, and would therefore provide better tools for prediction of drug induced liver damage.

6.2 *IN VIVO* STUDIES – ALCOHOL HEPATOTOXICITY

We observed in the studies focusing on development of alcoholic liver damage *in vivo* that:

- During the initial phase of ALD in rats we observe complicated patterns of cytokine and chemokine expression which fluctuate in relation to fluctuating levels of blood EtOH. This indicates that their mRNA expression may be directly influenced by absolute EtOH concentration, regardless of duration of EtOH treatment.

- Complex patterns of cytokine and chemokine mRNA expression are also seen in relation to duration of EtOH treatment. A strong initial Th1 response, on the levels of cytokine mRNAs as well as transcription factors, to be followed by a downregulation. Th2 response is down regulated by EtOH over the entire study period.

- The antioxidant NAC may decrease severity of liver damage as seen by significantly lower ALT levels and lower total pathology score in animals given EtOH + NAC in comparison to EtOH only.

- NAC can reduce ethanol-induced formation of protein adducts of lipid peroxidation products like MDA and HNE. Also, NAC decreases antibody formation against neo-antigens formed by MDA, HNE and HER protein adducts.

- The expression of the human form of CYP2E1 in a transgenic mouse model may significantly increase levels of pathological changes as determined by total pathology score. PCA analysis indicated that para-nitrophenol activity, mirroring CYP2E1 activity, was the factor most influencing total pathology score.

- When comparing transgenic EtOH treated animals to non-transgenic EtOH treated, we showed that several genes previously known to be associated with ALD development showed significant changes in expression between the two groups. For example TNFα induced increases in expression. Also, cytokeratins 8 and 18 known to be of importance for formation of Mallory bodies were shown to correlate to total pathology.

In summary, our studies show that the pattern of expression of inflammatory mediators early in ALD development is highly complex. We show that decreasing oxidative stress by introducing an antioxidant may counteract some of the detrimental features of ALD development. While overexpression of an enzyme known to generate reactive oxygen species during metabolism of substrate may aggravate pathology. These studies can hopefully be a basis for further detailed studies on the early inflammatory signs of ALD and of the mechanisms by which oxidative stress influences liver pathology.

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8 REFERENCES

Abo, T., H. Watanabe, et al. (1994). "Extrathymic pathways of T-cell differentiation in the liver and other organs." International Reviews of Immunology **11**(1): 61-102.

Afkarian, M., J. R. Sedy, et al. (2002). "T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells.[see comment]." Nature Immunology **3**(6): 549-57.

Ahmed, A. E., J. Aronson, et al. (2000). "Induction of oxidative stress and TNF-alpha secretion by dichloroacetonitrile, a water disinfectant by-product, as possible mediators of apoptosis or necrosis in a murine macrophage cell line (RAW)." Toxicology in Vitro **14**(3): 199-210.

Alabraba, E. B., V. Lai, et al. (2008). "Coculture of human liver macrophages and cholangiocytes leads to CD40-dependent apoptosis and cytokine secretion." Hepatology **47**(2): 552-62.

Albano, E. (2006). "Alcohol, oxidative stress and free radical damage." Proceedings of the Nutrition Society **65**(3): 278-90.

Albano, E., P. Clot, et al. (1996). "Role of cytochrome P4502E1-dependent formation of hydroxyethyl free radical in the development of liver damage in rats intragastrically fed with ethanol." Hepatology **23**(1): 155-63.

Albano, E., S. W. French, et al. (1999). "Hydroxyethyl radicals in ethanol hepatotoxicity." Frontiers in Bioscience **4**: D533-40.

Albano, E., E. Mottaran, et al. (2005). "Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis." Gut **54**(7): 987-93.

Almdal, T. P. and T. I. Sorensen (1991). "Incidence of parenchymal liver diseases in Denmark, 1981 to 1985: analysis of hospitalization registry data. The Danish Association for the Study of the Liver." Hepatology **13**(4): 650-5.

Al-Salman, J., H. Arjomand, et al. (2000). "Hepatocellular injury in a patient receiving rosiglitazone. A case report." Ann Intern Med **132**(2): 121-4.

Asahina, K., K. Teramoto, et al. (2006). "Embryonic stem cells: hepatic differentiation and regenerative medicine for the treatment of liver disease." Current Stem Cell Research & Therapy **1**(2): 139-56.

Aste-Amezaga, M., X. Ma, et al. (1998). "Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10.[erratum appears in J Immunol 2000 May 15;164(10):5330]." Journal of Immunology **160**(12): 5936-44.

Badger, T. M., J. O. Hoog, et al. (2000). "Cyclic expression of class I alcohol dehydrogenase in male rats treated with ethanol." Biochemical & Biophysical Research Communications **274**(3): 684-8.

Badger, T. M., M. J. Ronis, et al. (1993). "Pulsatile blood alcohol and CYP2E1 induction during chronic alcohol infusions in rats." Alcohol **10**(6): 453-7.

Badger, T. M., M. J. Ronis, et al. (1995). "Inhibition of CYP2E1 activity does not abolish pulsatile urine alcohol concentrations during chronic alcohol infusions." European Journal of Biochemistry **230**(3): 914-9.

Badger, T. M., M. J. Ronis, et al. (1993). "Effects of chronic ethanol on growth hormone secretion and hepatic cytochrome P450 isozymes of the rat." Journal of Pharmacology & Experimental Therapeutics **264**(1): 438-47.

Bai, J. and A. I. Cederbaum (2001). "Mitochondrial catalase and oxidative injury." Biological Signals & Receptors **10**(3-4): 189-99.

Bai, J. and A. I. Cederbaum (2003). "Catalase protects HepG2 cells from apoptosis induced by DNA-damaging agents by accelerating the degradation of p53." Journal of Biological Chemistry **278**(7): 4660-7.

Bai, J. and A. I. Cederbaum (2006). "Adenovirus-mediated expression of CYP2E1 produces liver toxicity in mice." Toxicological Sciences **91**(2): 365-71.

Bai, J., A. M. Rodriguez, et al. (1999). "Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury." Journal of Biological Chemistry **274**(37): 26217-24.

Banas, A., Y. Yamamoto, et al. (2007). "Stem cell plasticity: learning from hepatogenic differentiation strategies." Developmental Dynamics **236**(12): 3228-41.
Bardag-Gorce, F., B. A. French, et al. (2006). "CYP2E1 induced by ethanol causes oxidative stress, proteasome inhibition and cytokeratin aggresome (Mallory body-like) formation." Experimental & Molecular Pathology **81**(3): 191-201.
Baudoin, R., A. Corlu, et al. (2007). "Trends in the development of microfluidic cell biochips for in vitro hepatotoxicity." Toxicology in Vitro **21**(4): 535-44.
Baumgardner, J. N., K. Shankar, et al. (2007). "Undernutrition enhances alcohol-induced hepatocyte proliferation in the liver of rats fed via total enteral nutrition." American Journal of Physiology - Gastrointestinal & Liver Physiology **293**(1): G355-64.
Beckett, A. G., A. V. Livingstone, et al. (1961). "Acute alcoholic hepatitis." British Medical Journal **2**(5260): 1113-9.
Bellentani, S., G. Saccoccio, et al. (1997). "Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group.[see comment]." Gut **41**(6): 845-50.
Bernhardt, R. (2006). "Cytochromes P450 as versatile biocatalysts." Journal of Biotechnology **124**(1): 128-45.
Board, P., A. Blackburn, et al. (1998). "Polymorphism of phase II enzymes: identification of new enzymes and polymorphic variants by database analysis." Toxicology Letters **102-103**: 149-54.
Bonfanti, P., S. Landonio, et al. (2001). "Risk factors for hepatotoxicity in patients treated with highly active antiretroviral therapy." Journal of Acquired Immune Deficiency Syndromes: JAIDS **27**(3): 316-8.
Bova, M. P., D. Tam, et al. (2005). "Troglitazone induces a rapid drop of mitochondrial membrane potential in liver HepG2 cells." Toxicology Letters **155**(1): 41-50.
Bowen, D. G., G. W. McCaughan, et al. (2005). "Intrahepatic immunity: a tale of two sites?" Trends in Immunology **26**(10): 512-7.
Bowen, D. G., A. Warren, et al. (2002). "Cytokine-dependent bystander hepatitis due to intrahepatic murine CD8 T-cell activation by bone marrow-derived cells." Gastroenterology **123**(4): 1252-64.
Brady, M. T., B. P. Mahon, et al. (1998). "Pertussis infection and vaccination induces Th1 cells." Immunology Today **19**(11): 534.
Breiner, K. M., H. Schaller, et al. (2001). "Endothelial cell-mediated uptake of a hepatitis B virus: a new concept of liver targeting of hepatotropic microorganisms." Hepatology **34**(4 Pt 1): 803-8.
Burdin, N., L. Brossay, et al. (1998). "Selective ability of mouse CD1 to present glycolipids: alpha-galactosylceramide specifically stimulates V alpha 14+ NK T lymphocytes." Journal of Immunology **161**(7): 3271-81.
Burke, Z. D., S. Thowfeequ, et al. (2006). "Liver specification: a new role for Wnts in liver development." Current Biology **16**(17): R688-90.
Bykov, I., P. Ylipaasto, et al. (2003). "Phagocytosis and LPS-stimulated production of cytokines and prostaglandin E2 is different in Kupffer cells isolated from the periportal or perivenous liver region." Scandinavian Journal of Gastroenterology **38**(12): 1256-61.
Canning, C., M. O'Brien, et al. (2006). "Liver immunity and tumour surveillance." Immunology Letters **107**(2): 83-8.
Cao, Q., R. Batey, et al. (1998). "IL-6, IFN-gamma and TNF-alpha production by liver-associated T cells and acute liver injury in rats administered concanavalin A." Immunology & Cell Biology **76**(6): 542-9.
Carriere, A., M. C. Carmona, et al. (2004). "Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: a mechanism for hypoxia-dependent effect." Journal of Biological Chemistry **279**(39): 40462-9.
Cederbaum, A. I. (2006). "CYP2E1--biochemical and toxicological aspects and role in alcohol-induced liver injury." Mount Sinai Journal of Medicine **73**(4): 657-72.
Cederbaum, A. I., D. Wu, et al. (2001). "CYP2E1-dependent toxicity and oxidative stress in HepG2 cells." Free Radical Biology & Medicine **31**(12): 1539-43.
Charpentier, B., D. Franco, et al. (1984). "Deficient natural killer cell activity in alcoholic cirrhosis." Clinical & Experimental Immunology **58**(1): 107-15.

Cheung, C., T. E. Akiyama, et al. (2003). "Hepatic expression of cytochrome P450s in hepatocyte nuclear factor 1-alpha (HNF1alpha)-deficient mice." Biochemical Pharmacology **66**(10): 2011-20.
Cheung, C., A. M. Yu, et al. (2005). "The cyp2e1-humanized transgenic mouse: role of cyp2e1 in acetaminophen hepatotoxicity." Drug Metabolism & Disposition **33**(3): 449-57.
Cho, H. Y., S. P. Reddy, et al. (2006). "Nrf2 defends the lung from oxidative stress." Antioxidants & Redox Signaling **8**(1-2): 76-87.
Clot, P., E. Albano, et al. (1996). "Cytochrome P4502E1 hydroxyethyl radical adducts as the major antigen in autoantibody formation among alcoholics." Gastroenterology **111**(1): 206-16.
Collom, S. L., A. P. Jamakhandi, et al. (2007). "CYP2E1 active site residues in substrate recognition sequence 5 identified by photoaffinity labeling and homology modeling." Archives of Biochemistry & Biophysics **459**(1): 59-69.
Cowdery, J. S., J. H. Chace, et al. (1996). "Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides." Journal of Immunology **156**(12): 4570-5.
Crews, F., K. Nixon, et al. (2006). "BHT blocks NF-kappaB activation and ethanol-induced brain damage." Alcoholism: Clinical & Experimental Research **30**(11): 1938-49.
Crews, F. T., R. Bechara, et al. (2006). "Cytokines and alcohol." Alcoholism: Clinical & Experimental Research **30**(4): 720-30.
Cunningham, C. C. and C. G. Van Horn (2003). "Energy availability and alcohol-related liver pathology." Alcohol Res Health **27**(4): 291-9.
Czaja, M. J., A. Geerts, et al. (1994). "Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease." Journal of Leukocyte Biology **55**(1): 120-6.
Dambach, D. M., B. A. Andrews, et al. (2005). "New technologies and screening strategies for hepatotoxicity: use of in vitro models." Toxicologic Pathology **33**(1): 17-26.
Day, C. P. and O. F. James (1998). "Steatohepatitis: a tale of two "hits"?" Gastroenterology **114**(4): 842-5.
de Abajo, F. J., D. Montero, et al. (2004). "Acute and clinically relevant drug-induced liver injury: a population based case-control study." British Journal of Clinical Pharmacology **58**(1): 71-80.
de la, M. H. P., C. S. Lieber, et al. (2001). "Models of alcoholic liver disease in rodents: a critical evaluation." Alcoholism: Clinical & Experimental Research **25**(5 Suppl ISBRA): 254S-261S.
DeCarli, L. M. and C. S. Lieber (1967). "Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet." Journal of Nutrition **91**(3): 331-6.
Del Rio, L., A. J. Buendia, et al. (2001). "Endogenous interleukin-12 is not required for resolution of Chlamydophila abortus (Chlamydia psittaci serotype 1) infection in mice." Infection & Immunity **69**(8): 4808-15.
Dominguez-Santalla, M. J., C. Vidal, et al. (2001). "Increased serum IgE in alcoholics: relationship with Th1/Th2 cytokine production by stimulated blood mononuclear cells." Alcoholism: Clinical & Experimental Research **25**(8): 1198-205.
Dory, D., H. Echchannaoui, et al. (2003). "Generation and functional characterization of a clonal murine periportal Kupffer cell line from H-2Kb -tsA58 mice." Journal of Leukocyte Biology **74**(1): 49-59.
Driscoll, K. E. (2000). "TNFalpha and MIP-2: role in particle-induced inflammation and regulation by oxidative stress." Toxicology Letters **112-113**: 177-83.
Dupont, I., D. Lucas, et al. (1998). "Cytochrome P4502E1 inducibility and hydroxyethyl radical formation among alcoholics." Journal of Hepatology **28**(4): 564-71.
Ek, M., T. Soderdahl, et al. (2007). "Expression of drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells." Biochemical Pharmacology **74**(3): 496-503.

Ekstrom, G. and M. Ingelman-Sundberg (1989). "Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1)." Biochemical Pharmacology **38**(8): 1313-9.
Eliasson, E., I. Johansson, et al. (1988). "Ligand-dependent maintenance of ethanol-inducible cytochrome P-450 in primary rat hepatocyte cell cultures." Biochemical & Biophysical Research Communications **150**(1): 436-43.
Eliasson, E., I. Johansson, et al. (1990). "Substrate-, hormone-, and cAMP-regulated cytochrome P450 degradation.[erratum appears in Proc Natl Acad Sci U S A 1990 Jul;87(13):5232]." Proceedings of the National Academy of Sciences of the United States of America **87**(8): 3225-9.
Erhardt, A., M. Biburger, et al. (2007). "IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin A-induced liver injury in mice." Hepatology **45**(2): 475-85.
Fang, C., K. O. Lindros, et al. (1998). "Zonated expression of cytokines in rat liver: effect of chronic ethanol and the cytochrome P450 2E1 inhibitor, chlormethiazole." Hepatology **27**(5): 1304-10.
Fausto, N. (2005). "Tweaking liver progenitor cells." Nature Medicine **11**(10): 1053-4.
Franco, R., O. J. Schoneveld, et al. (2007). "The central role of glutathione in the pathophysiology of human diseases." Archives of Physiology & Biochemistry **113**(4-5): 234-58.
Friedman, S. L. (2008). "Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver." Physiological Reviews **88**(1): 125-72.
Friedman, S. L. and M. J. Arthur (1989). "Activation of cultured rat hepatic lipocytes by Kupffer cell conditioned medium. Direct enhancement of matrix synthesis and stimulation of cell proliferation via induction of platelet-derived growth factor receptors." Journal of Clinical Investigation **84**(6): 1780-5.
Gao, J., L. Ann Garulacan, et al. (2004). "Identification of in vitro protein biomarkers of idiosyncratic liver toxicity." Toxicology in Vitro **18**(4): 533-41.
Garcia Rodriguez, L. A., A. Ruigomez, et al. (1997). "A review of epidemiologic research on drug-induced acute liver injury using the general practice research data base in the United Kingdom." Pharmacotherapy **17**(4): 721-8.
Gebhardt, R. and D. Mecke (1983). "Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells in situ and in primary culture." EMBO Journal **2**(4): 567-70.
Gehring, S., E. M. Dickson, et al. (2006). "Kupffer cells abrogate cholestatic liver injury in mice." Gastroenterology **130**(3): 810-22.
Geissmann, F., T. O. Cameron, et al. (2005). "Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids." Plos Biology **3**(4): e113.
Gergel, D., V. Misik, et al. (1997). "Inhibition of rat and human cytochrome P4502E1 catalytic activity and reactive oxygen radical formation by nitric oxide." Archives of Biochemistry & Biophysics **337**(2): 239-50.
Girouard, L., P. Mandrekar, et al. (1998). "Regulation of monocyte interleukin-12 production by acute alcohol: a role for inhibition by interleukin-10." Alcoholism: Clinical & Experimental Research **22**(1): 211-6.
Gong, P. and A. I. Cederbaum (2006). "Transcription factor Nrf2 protects HepG2 cells against CYP2E1 plus arachidonic acid-dependent toxicity." Journal of Biological Chemistry **281**(21): 14573-9.
Gonzalez, F. J. (2005). "Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1." Mutation Research **569**(1-2): 101-10.
Gonzalez, F. J. (2007). "The 2006 Bernard B. Brodie Award Lecture. Cyp2e1." Drug Metabolism & Disposition **35**(1): 1-8.
Gonzalez-Quintela, A., J. Garcia, et al. (2006). "Serum cytokeratins in alcoholic liver disease: contrasting levels of cytokeratin-18 and cytokeratin-19." Alcohol **38**(1): 45-9.
Gonzalez-Quintela, A., C. Vidal, et al. (1999). "Serum cytokines and increased total serum IgE in alcoholics." Annals of Allergy, Asthma, & Immunology **83**(1): 61-7.

Gonzalez-Reimers, E., E. Garcia-Valdecasas-Campelo, et al. (2007). "Pro-inflammatory cytokines in stable chronic alcoholics: relationship with fat and lean mass." Food & Chemical Toxicology **45**(6): 904-9.
Gregory, S. H., L. K. Barczynski, et al. (1992). "Effector function of hepatocytes and Kupffer cells in the resolution of systemic bacterial infections." Journal of Leukocyte Biology **51**(4): 421-4.
Gripon, P., S. Rumin, et al. (2002). "Infection of a human hepatoma cell line by hepatitis B virus." Proc Natl Acad Sci U S A **99**(24): 15655-60.
Grisham, J. W. (1979). "Use of hepatic cell cultures to detect and evaluate the mechanisms of action of toxic chemicals." International Review of Experimental Pathology **20**: 123-210.
Grisham, J. W. (1983). "Cell types in rat liver cultures: their identification and isolation." Molecular & Cellular Biochemistry **53-54**(1-2): 23-33.
Groneberg, D. A., C. Grosse-Siestrup, et al. (2002). "In vitro models to study hepatotoxicity." Toxicologic Pathology **30**(3): 394-9.
Guengerich, F. P., D. H. Kim, et al. (1991). "Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects." Chemical Research in Toxicology **4**(2): 168-79.
Guillouzo, A., A. Corlu, et al. (2007). "The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics." Chemico-Biological Interactions **168**(1): 66-73.
Hallows, K. R. and R. S. Frank (1992). "Changes in mechanical properties with DMSO-induced differentiation of HL-60 cells." Biorheology **29**(2-3): 295-309.
Hambleton, J., S. L. Weinstein, et al. (1996). "Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages." Proceedings of the National Academy of Sciences of the United States of America **93**(7): 2774-8.
Hendriks, H. F., A. Brouwer, et al. (1990). "Isolation, purification, and characterization of liver cell types." Methods in Enzymology **190**: 49-58.
Hendriks, H. F., W. A. Verhoofstad, et al. (1985). "Perisinusoidal fat-storing cells are the main vitamin A storage sites in rat liver." Experimental Cell Research **160**(1): 138-49.
Hetherington, S., A. R. Hughes, et al. (2002). "Genetic variations in HLA-B region and hypersensitivity reactions to abacavir." Lancet **359**(9312): 1121-2.
Hu, Y., J. Hakkola, et al. (1999). "Structural and functional characterization of the 5'-flanking region of the rat and human cytochrome P450 2E1 genes: identification of a polymorphic repeat in the human gene." Biochemical & Biophysical Research Communications **263**(2): 286-93.
Hu, Y., M. Oscarson, et al. (1997). "Genetic polymorphism of human CYP2E1: characterization of two variant alleles." Molecular Pharmacology **51**(3): 370-6.
Hussaini, S. H. and E. A. Farrington (2007). "Idiosyncratic drug-induced liver injury: an overview." Expert Opinion on Drug Safety **6**(6): 673-84.
Ingelman-Sundberg, M. (2001). "Implications of polymorphic cytochrome p450-dependent drug metabolism for drug development." Drug Metabolism & Disposition **29**(4 Pt 2): 570-3.
Ingelman-Sundberg, M. (2004). "Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms." Naunyn-Schmiedebergs Archives of Pharmacology **369**(1): 89-104.
Ingelman-Sundberg, M. and I. Johansson (1984). "Mechanisms of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450." Journal of Biological Chemistry **259**(10): 6447-58.
Ingelman-Sundberg, M., I. Johansson, et al. (1988). "Centrilobular expression of ethanol-inducible cytochrome P-450 (IIE1) in rat liver." Biochemical & Biophysical Research Communications **157**(1): 55-60.
Ingelman-Sundberg, M., I. Johansson, et al. (1993). "Ethanol-inducible cytochrome P4502E1: genetic polymorphism, regulation, and possible role in the etiology of alcohol-induced liver disease." Alcohol **10**(6): 447-52.
Isayama, F., M. Froh, et al. (2004). "TNF alpha-induced Ras activation due to ethanol promotes hepatocyte proliferation independently of liver injury in the mouse." Hepatology **39**(3): 721-31.

Ishii, T., K. Itoh, et al. (2000). "Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages." Journal of Biological Chemistry **275**(21): 16023-9.
Ishiyama, K., H. Ohdan, et al. (2006). "Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans." Hepatology **43**(2): 362-72.
Itoh, Y., T. Okanoue, et al. (1992). "Functional heterogeneity of rat liver macrophages: interleukin-1 secretion and Ia antigen expression in contrast with phagocytic activity." Liver **12**(1): 26-33.
Jaeschke, H. (2007). "Troglitazone hepatotoxicity: are we getting closer to understanding idiosyncratic liver injury?[comment]." Toxicological Sciences **97**(1): 1-3.
Jayatilleke, A. and S. Shaw (1998). "Stimulation of monocyte interleukin-8 by lipid peroxidation products: a mechanism for alcohol-induced liver injury." Alcohol **16**(2): 119-23.
Jeejeebhoy, K. and M. J. Phillips (1976). "Isolated mammalian hepatocytes in culture." Gastroenterology **71**(6): 1086-96.
Johansson, I., G. Ekstrom, et al. (1988). "Ethanol-, fasting-, and acetone-inducible cytochromes P-450 in rat liver: regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies." Biochemistry **27**(6): 1925-34.
Johansson, I. and M. Ingelman-Sundberg (1985). "Carbon tetrachloride-induced lipid peroxidation dependent on an ethanol-inducible form of rabbit liver microsomal cytochrome P-450." FEBS Letters **183**(2): 265-9.
Johansson, I., K. O. Lindros, et al. (1990). "Transcriptional control of CYP2E1 in the perivenous liver region and during starvation." Biochemical & Biophysical Research Communications **173**(1): 331-8.
Jokelainen, K., L. A. Reinke, et al. (2001). "Nf-kappab activation is associated with free radical generation and endotoxemia and precedes pathological liver injury in experimental alcoholic liver disease." Cytokine **16**(1): 36-9.
Kamimura, S. and H. Tsukamoto (1995). "Cytokine gene expression by Kupffer cells in experimental alcoholic liver disease." Hepatology **22**(4 Pt 1): 1304-9.
Kanebratt, K. P. and T. B. Andersson (2008). "HepaRG cells as an in vitro model for evaluation of cytochrome P450 induction in humans." Drug Metabolism & Disposition **36**(1): 137-45.
Kaplowitz, N. (2000). "Mechanisms of liver cell injury." Journal of Hepatology **32**(1 Suppl): 39-47.
Kaplowitz, N. (2005). "Idiosyncratic drug hepatotoxicity." Nature Reviews. Drug Discovery **4**(6): 489-99.
Karaa, A. T., Kyle J.; McKillop, Iain H.; Clemens, Mark G.; Schrum, Laura W. (2008). "S-adenosyl-L-methionine attenuates oxidative stress and hepatic stellate cell activation in an ethanol-LPS-induced fibrotic rat model." Shock **Published Ahead of Print**.
Karlgren, M., A. Gomez, et al. (2006). "Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1." Biochemical & Biophysical Research Communications **341**(2): 451-8.
Kenai, H., G. Matsuzaki, et al. (1995). "Precursor cells to CD3-intermediate (CD3int) liver mononuclear cells in the adult liver: further evidence for the extrathymic development of CD3int liver mononuclear cells." European Journal of Immunology **25**(12): 3365-9.
Kindmark, A., A. Jawaid, et al. (2007). "Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis." Pharmacogenomics J.
Kishore, R., M. R. McMullen, et al. (2001). "Stabilization of tumor necrosis factor alpha mRNA by chronic ethanol: role of A + U-rich elements and p38 mitogen-activated protein kinase signaling pathway." Journal of Biological Chemistry **276**(45): 41930-7.
Kmiec, Z. (2001). "Cooperation of liver cells in health and disease." Advances in Anatomy, Embryology & Cell Biology **161**: III-XIII.

Knook, D. L., N. Blansjaar, et al. (1977). "Isolation and characterization of Kupffer and endothelial cells from the rat liver." Experimental Cell Research **109**(2): 317-29.
Knook, D. L., A. M. Seffelaar, et al. (1982). "Fat-storing cells of the rat liver. Their isolation and purification." Experimental Cell Research **139**(2): 468-71.
Koch, O. R., T. Galeotti, et al. (1991). "Alcohol-induced oxidative stress in rat liver." Xenobiotica **21**(8): 1077-84.
Kono, H., B. U. Bradford, et al. (1999). "CYP2E1 is not involved in early alcohol-induced liver injury." American Journal of Physiology **277**(6 Pt 1): G1259-67.
Kopf, M., G. Le Gros, et al. (1993). "Disruption of the murine IL-4 gene blocks Th2 cytokine responses." Nature **362**(6417): 245-8.
Korourian, S., R. Hakkak, et al. (1999). "Diet and risk of ethanol-induced hepatotoxicity: carbohydrate-fat relationships in rats." Toxicological Sciences **47**(1): 110-7.
Kumashiro, Y., K. Asahina, et al. (2005). "Enrichment of hepatocytes differentiated from mouse embryonic stem cells as a transplantable source." Transplantation **79**(5): 550-7.
Lako, M., S. Lindsay, et al. (2001). "Characterisation of Wnt gene expression during the differentiation of murine embryonic stem cells in vitro: role of Wnt3 in enhancing haematopoietic differentiation." Mechanisms of Development **103**(1-2): 49-59.
Laso, F. J., C. Iglesias-Osma, et al. (1999). "Chronic alcoholism is associated with an imbalanced production of Th-1/Th-2 cytokines by peripheral blood T cells.[see comment]." Alcoholism: Clinical & Experimental Research **23**(8): 1306-11.
Laso, F. J., P. Lapena, et al. (1997). "Alterations in tumor necrosis factor-alpha, interferon-gamma, and interleukin-6 production by natural killer cell-enriched peripheral blood mononuclear cells in chronic alcoholism: relationship with liver disease and ethanol intake." Alcoholism: Clinical & Experimental Research **21**(7): 1226-31.
Laso, F. J., J. I. Madruga, et al. (1997). "Abnormalities of peripheral blood T lymphocytes and natural killer cells in alcoholic hepatitis persist after a 3-month withdrawal period." Alcoholism: Clinical & Experimental Research **21**(4): 672-6.
Laso, F. J., J. M. Vaquero, et al. (2007). "Chronic alcohol consumption is associated with changes in the distribution, immunophenotype, and the inflammatory cytokine secretion profile of circulating dendritic cells." Alcoholism: Clinical & Experimental Research **31**(5): 846-54.
Latvala, J., J. Hietala, et al. (2005). "Immune Responses to Ethanol Metabolites and Cytokine Profiles Differentiate Alcoholics with or without Liver Disease." American Journal of Gastroenterology **100**(6): 1303-10.
Lauterburg, B. H., G. B. Corcoran, et al. (1983). "Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of acetaminophen in rats in vivo." Journal of Clinical Investigation **71**(4): 980-91.
Lazarou, J., B. H. Pomeranz, et al. (1998). "Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies." Jama **279**(15): 1200-5.
Le Jossic, C., D. Glaise, et al. (1996). "trans-Acting factors, detoxication enzymes and hepatitis B virus replication in a novel set of human hepatoma cell lines." Eur J Biochem **238**(2): 400-9.
Lee, W. C., M. C. Yu, et al. (2005). "Liver stellate cells suppress dendritic cells through IL-10." Transplantation Proceedings **37**(1): 10-1.
Lelbach, W. K. (1975). "Cirrhosis in the alcoholic and its relation to the volume of alcohol abuse." Annals of the New York Academy of Sciences **252**: 85-105.
Lemire, J. M., N. Shiojiri, et al. (1991). "Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine." American Journal of Pathology **139**(3): 535-52.
Leo, M. A. and C. S. Lieber (1983). "Hepatic fibrosis after long-term administration of ethanol and moderate vitamin A supplementation in the rat." Hepatology **3**(1): 1-11.

Letteron, P., B. Fromenty, et al. (1996). "Acute and chronic hepatic steatosis lead to in vivo lipid peroxidation in mice." Journal of Hepatology **24**(2): 200-8.
Levenberg, S., N. F. Huang, et al. (2003). "Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds." Proceedings of the National Academy of Sciences of the United States of America **100**(22): 12741-6.
Lewis, D. F., M. G. Bird, et al. (2000). "Molecular modelling of human CYP2E1 by homology with the CYP102 haemoprotein domain: investigation of the interactions of substrates and inhibitors within the putative active site of the human CYP2E1 isoform." Xenobiotica **30**(1): 1-25.
Lewis, D. F., B. G. Lake, et al. (2003). "Homology modelling of human CYP2E1 based on the CYP2C5 crystal structure: investigation of enzyme-substrate and enzyme-inhibitor interactions." Toxicology in Vitro **17**(1): 93-105.
Li, W. C., K. L. Ralphs, et al. (2007). "Keratinocyte serum-free medium maintains long-term liver gene expression and function in cultured rat hepatocytes by preventing the loss of liver-enriched transcription factors." International Journal of Biochemistry & Cell Biology **39**(3): 541-54.
Lickteig, A. J., C. D. Fisher, et al. (2007). "Genes of the antioxidant response undergo upregulation in a rodent model of nonalcoholic steatohepatitis." Journal of Biochemical & Molecular Toxicology **21**(4): 216-20.
Lieber, C. S. (1988). "The microsomal ethanol oxidizing system: its role in ethanol and xenobiotic metabolism." Biochemical Society Transactions **16**(3): 232-9.
Lieber, C. S. (1994). "Susceptibility to alcohol-related liver injury." Alcohol & Alcoholism. Supplement **2**: 315-26.
Lieber, C. S. (2004). "The discovery of the microsomal ethanol oxidizing system and its physiologic and pathologic role." Drug Metabolism Reviews **36**(3-4): 511-29.
Lieber, C. S. (2005). "Pathogenesis and treatment of alcoholic liver disease: progress over the last 50 years." Roczniki Akademii Medycznej W Bialymstoku **50**: 7-20.
Lieber, C. S., E. Rubin, et al. (1970). "Hepatic microsomal ethanol oxidizing system (MEOS): differentiation from alcohol dehydrogenase and NADPH oxidase." Biochemical & Biophysical Research Communications **40**(4): 858-65.
Lin, J., C. Handschin, et al. (2005). "Metabolic control through the PGC-1 family of transcription coactivators." Cell Metabolism **1**(6): 361-70.
Lindros, K. O. and H. A. Jarvelainen (1998). "A new oral low-carbohydrate alcohol liquid diet producing liver lesions: a preliminary account." Alcohol & Alcoholism **33**(4): 347-53.
Liu, S. Y. and F. J. Gonzalez (1995). "Role of the liver-enriched transcription factor HNF-1 alpha in expression of the CYP2E1 gene." DNA & Cell Biology **14**(4): 285-93.
Liu, Z. X. and N. Kaplowitz (2002). "Immune-mediated drug-induced liver disease." Clinics in Liver Disease **6**(3): 755-74.
Lowry, R. P., T. Takeuchi, et al. (1993). "Th2-like effectors may function as antigen-specific suppressor cells in states of transplantation tolerance." Transplantation Proceedings **25**(1 Pt 1): 324-6.
Luhe, A., L. Suter, et al. (2005). "Toxicogenomics in the pharmaceutical industry: hollow promises or real benefit?" Mutation Research **575**(1-2): 102-15.
Maher, J. J., C. Tzagarakis, et al. (1994). "Malondialdehyde stimulates collagen production by hepatic lipocytes only upon activation in primary culture." Alcohol & Alcoholism **29**(5): 605-10.
Mallal, S., D. Nolan, et al. (2002). "Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir.[see comment]." Lancet **359**(9308): 727-32.
Mallal, S., E. Phillips, et al. (2008). "HLA-B*5701 screening for hypersensitivity to abacavir." N Engl J Med **358**(6): 568-79.
Mandayam, S., M. M. Jamal, et al. (2004). "Epidemiology of alcoholic liver disease." Seminars in Liver Disease **24**(3): 217-32.
Marchand, L. L., G. R. Wilkinson, et al. (1999). "Genetic and dietary predictors of CYP2E1 activity: a phenotyping study in Hawaii Japanese using

chlorzoxazone." Cancer Epidemiology, Biomarkers & Prevention **8**(6): 495-500.
Mari, M., J. Bai, et al. (2002). "Toxicity by pyruvate in HepG2 cells depleted of glutathione: role of mitochondria." Free Radical Biology & Medicine **32**(1): 73-83.
Mari, M. and A. I. Cederbaum (2001). "Induction of catalase, alpha, and microsomal glutathione S-transferase in CYP2E1 overexpressing HepG2 cells and protection against short-term oxidative stress." Hepatology **33**(3): 652-61.
Mehal, W. Z., F. Azzaroli, et al. (2001). "Immunology of the healthy liver: old questions and new insights." Gastroenterology **120**(1): 250-60.
Meng, F. and C. A. Lowell (1997). "Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn." Journal of Experimental Medicine **185**(9): 1661-70.
Michalec, L., B. K. Choudhury, et al. (2002). "CCL7 and CXCL10 orchestrate oxidative stress-induced neutrophilic lung inflammation." Journal of Immunology **168**(2): 846-52.
Miller, M. S., M. R. Juchau, et al. (1996). "Drug metabolic enzymes in developmental toxicology." Fundamental & Applied Toxicology **34**(2): 165-75.
Misra, U. K., B. U. Bradford, et al. (1992). "Chronic ethanol treatment induces H2O2 production selectively in pericentral regions of the liver lobule." Alcoholism: Clinical & Experimental Research **16**(5): 839-42.
Mizuhara, H., E. O'Neill, et al. (1994). "T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6." Journal of Experimental Medicine **179**(5): 1529-37.
Mizuno, Y., H. Takada, et al. (2003). "Th1 and Th1-inducing cytokines in Salmonella infection." Clinical & Experimental Immunology **131**(1): 111-7.
Moore, T. J., M. R. Cohen, et al. (2007). "Serious adverse drug events reported to the Food and Drug Administration, 1998-2005." Archives of Internal Medicine **167**(16): 1752-9.
Morgan, K., S. W. French, et al. (2002). "Production of a cytochrome P450 2E1 transgenic mouse and initial evaluation of alcoholic liver damage." Hepatology **36**(1): 122-34.
Mosmann, T. R., H. Cherwinski, et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." Journal of Immunology **136**(7): 2348-57.
Mottaran, E., S. F. Stewart, et al. (2002). "Lipid peroxidation contributes to immune reactions associated with alcoholic liver disease." Free Radical Biology & Medicine **32**(1): 38-45.
Munthe-Kaas, A. C., T. Berg, et al. (1975). "Mass isolation and culture of rat kupffer cells." Journal of Experimental Medicine **141**(1): 1-10.
Munthe-Kaas, A. C., T. Berg, et al. (1976). "Distribution of lysosomal enzymes in different types of rat liver cells." Experimental Cell Research **99**(1): 146-54.
Murphy, K. M., W. Ouyang, et al. (2000). "Signaling and transcription in T helper development." Annual Review of Immunology **18**: 451-94.
Mustafa, S. B. and M. S. Olson (1998). "Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP." Journal of Biological Chemistry **273**(9): 5073-80.
Nakamichi, I., S. Hatakeyama, et al. (2002). "Formation of Mallory body-like inclusions and cell death induced by deregulated expression of keratin 18." Molecular Biology of the Cell **13**(10): 3441-51.
Nakamichi, I., D. M. Toivola, et al. (2005). "Keratin 8 overexpression promotes mouse Mallory body formation." Journal of Cell Biology **171**(6): 931-7.
Nanji, A. A. and S. W. French (1988). "Effect of different dietary fats on ethanol metabolism: implications for pathogenesis of experimental alcoholic liver disease." International Journal for Vitamin & Nutrition Research **58**(4): 475-6.
Nardone, G., M. Romano, et al. (1996). "Activation of fetal promoters of insulinlike growth factors II gene in hepatitis C virus-related chronic hepatitis, cirrhosis, and hepatocellular carcinoma." Hepatology **23**(6): 1304-12.
Nelson, D. R., D. C. Zeldin, et al. (2004). "Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature

recommendations for genes, pseudogenes and alternative-splice variants." Pharmacogenetics **14**(1): 1-18.
Niemela, O., S. Parkkila, et al. (2000). "Cytochromes P450 2A6, 2E1, and 3A and production of protein-aldehyde adducts in the liver of patients with alcoholic and non-alcoholic liver diseases." Journal of Hepatology **33**(6): 893-901.
Nieto, N. and A. I. Cederbaum (2003). "Increased Sp1-dependent transactivation of the LAMgamma 1 promoter in hepatic stellate cells co-cultured with HepG2 cells overexpressing cytochrome P450 2E1." Journal of Biological Chemistry **278**(17): 15360-72.
Nilsson, R., V. B. Bajic, et al. (2006). "Transcriptional network dynamics in macrophage activation." Genomics **88**(2): 133-42.
Norris, S., C. Collins, et al. (1998). "Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes." Journal of Hepatology **28**(1): 84-90.
Norris W, P. A., Lewis JH (2008). "Drug-induced liver injury in 2007." Curr Opin Gastroenterol. **24**(3): 287-97.
Novobrantseva, T. I., G. R. Majeau, et al. (2005). "Attenuated liver fibrosis in the absence of B cells.[see comment]." Journal of Clinical Investigation **115**(11): 3072-82.
Ong, M. M., C. Latchoumycandane, et al. (2007). "Troglitazone-induced hepatic necrosis in an animal model of silent genetic mitochondrial abnormalities.[see comment]." Toxicological Sciences **97**(1): 205-13.
Overturf, K., M. Al-Dhalimy, et al. (1999). "The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion." American Journal of Pathology **155**(6): 2135-43.
Park, B. K., M. Pirmohamed, et al. (1995). "The role of cytochrome P450 enzymes in hepatic and extrahepatic human drug toxicity." Pharmacology & Therapeutics **68**(3): 385-424.
Parola, M., M. Pinzani, et al. (1996). "Induction of procollagen type I gene expression and synthesis in human hepatic stellate cells by 4-hydroxy-2,3-nonenal and other 4-hydroxy-2,3-alkenals is related to their molecular structure." Biochemical & Biophysical Research Communications **222**(2): 261-4.
Peng, Y. and M. M. Murr (2007). "Establishment of immortalized rat Kupffer cell lines." Cytokine **37**(3): 185-91.
Phillips, M. J., M. Oda, et al. (1974). "Ultrastructural and functional studies of cultured hepatocytes." Laboratory Investigation **31**(5): 533-42.
Pi, J., Q. Zhang, et al. (2008). "Activation of Nrf2-mediated oxidative stress response in macrophages by hypochlorous acid." Toxicology & Applied Pharmacology **226**(3): 236-43.
Plescia, C., C. Rogler, et al. (2001). "Genomic expression analysis implicates Wnt signaling pathway and extracellular matrix alterations in hepatic specification and differentiation of murine hepatic stem cells." Differentiation **68**(4-5): 254-69.
Pratt, M. A., C. Crippen, et al. (1998). "Deregulated expression of the retinoid X receptor alpha prevents muscle differentiation in P19 embryonal carcinoma cells." Cell Growth & Differentiation **9**(9): 713-22.
Pruett, S. B., R. Fan, et al. (2005). "Differences in IL-10 and IL-12 production patterns and differences in the effects of acute ethanol treatment on macrophages in vivo and in vitro." Alcohol **37**(1): 1-8.
Reinecke, F., O. Levanets, et al. (2006). "Metallothionein isoform 2A expression is inducible and protects against ROS-mediated cell death in rotenone-treated HeLa cells." Biochemical Journal **395**(2): 405-15.
Rendic, S. and F. J. Di Carlo (1997). "Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors." Drug Metabolism Reviews **29**(1-2): 413-580.
Rigamonti, C., E. Mottaran, et al. (2003). "Moderate alcohol consumption increases oxidative stress in patients with chronic hepatitis C." Hepatology **38**(1): 42-9.
Robinson, D. S., Q. Hamid, et al. (1992). "Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma." New England Journal of Medicine **326**(5): 298-304.

Romagnani, S. (1995). "Biology of human TH1 and TH2 cells." Journal of Clinical Immunology **15**(3): 121-9.
Romagnani, S. (1996). "Th1 and Th2 in human diseases." Clinical Immunology & Immunopathology **80**(3 Pt 1): 225-35.
Ronis, M. J., R. Hakkak, et al. (2004). "Alcoholic liver disease in rats fed ethanol as part of oral or intragastric low-carbohydrate liquid diets." Experimental Biology & Medicine **229**(4): 351-60.
Roskams, T. (2006). "Different types of liver progenitor cells and their niches.[comment]." Journal of Hepatology **45**(1): 1-4.
Ryan, D. E., D. R. Koop, et al. (1986). "Evidence that isoniazid and ethanol induce the same microsomal cytochrome P-450 in rat liver, an isozyme homologous to rabbit liver cytochrome P-450 isozyme 3a." Archives of Biochemistry & Biophysics **246**(2): 633-44.
Sakurai, F., T. Terada, et al. (2002). "The role of tissue macrophages in the induction of proinflammatory cytokine production following intravenous injection of lipoplexes." Gene Therapy **9**(16): 1120-6.
Sampey, B. P., S. Korourian, et al. (2003). "Immunohistochemical characterization of hepatic malondialdehyde and 4-hydroxynonenal modified proteins during early stages of ethanol-induced liver injury." Alcoholism: Clinical & Experimental Research **27**(6): 1015-22.
Schoepfer, A. M., A. Engel, et al. (2007). "Herbal does not mean innocuous: ten cases of severe hepatotoxicity associated with dietary supplements from Herbalife products.[see comment]." Journal of Hepatology **47**(4): 521-6.
Schumann, J., D. Wolf, et al. (2000). "Importance of Kupffer cells for T-cell-dependent liver injury in mice." American Journal of Pathology **157**(5): 1671-83.
Seki, S., Y. Habu, et al. (2000). "The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses." Immunological Reviews **174**: 35-46.
Seki, S., S. Osada, et al. (1998). "Role of liver NK cells and peritoneal macrophages in gamma interferon and interleukin-10 production in experimental bacterial peritonitis in mice." Infection & Immunity **66**(11): 5286-94.
Sekine, S., B. Y. Lan, et al. (2006). "Liver-specific loss of beta-catenin blocks glutamine synthesis pathway activity and cytochrome p450 expression in mice.[see comment]." Hepatology **43**(4): 817-25.
Sgro, C., F. Clinard, et al. (2002). "Incidence of drug-induced hepatic injuries: a French population-based study.[see comment]." Hepatology **36**(2): 451-5.
Shah, N. L. and F. D. Gordon (2007). "N-acetylcysteine for acetaminophen overdose: when enough is enough.[comment]." Hepatology **46**(3): 939-41.
Sirica, A. E. and H. C. Pitot (1979). "Drug metabolism and effects of carcinogens in cultured hepatic cells." Pharmacological Reviews **31**(3): 205-28.
Sleyster, E. C. and D. L. Knook (1982). "Relation between localization and function of rat liver Kupffer cells." Laboratory Investigation **47**(5): 484-90.
Smedsrod, B., H. Pertoft, et al. (1985). "Functional and morphological characterization of cultures of Kupffer cells and liver endothelial cells prepared by means of density separation in Percoll, and selective substrate adherence." Cell & Tissue Research **241**(3): 639-49.
Smith, M. T. and E. D. Wills (1981). "Effects of dietary lipid and phenobarbitone on the distribution and concentration of cytochrome P-450 in the liver studied by quantitative cytochemistry." FEBS Letters **127**(1): 33-6.
Song, B. J., T. Matsunaga, et al. (1987). "Stabilization of cytochrome P450j messenger ribonucleic acid in the diabetic rat." Molecular Endocrinology **1**(8): 542-7.
Song, B. J., R. L. Veech, et al. (1989). "Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization." Journal of Biological Chemistry **264**(6): 3568-72.
Soto-Gutierrez, A., N. Navarro-Alvarez, et al. (2007). "Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines." Nature Protocols **2**(2): 347-56.
Speisky, H., A. MacDonald, et al. (1985). "Increased loss and decreased synthesis of hepatic glutathione after acute ethanol administration. Turnover studies." Biochemical Journal **225**(3): 565-72.

Steiner, G., L. Suter, et al. (2004). "Discriminating different classes of toxicants by transcript profiling." Environmental Health Perspectives **112**(12): 1236-48.
Stewart, S. F., M. Vidali, et al. (2004). "Oxidative stress as a trigger for cellular immune responses in patients with alcoholic liver disease." Hepatology **39**(1): 197-203.
Strnad, P., R. Windoffer, et al. (2002). "Induction of rapid and reversible cytokeratin filament network remodeling by inhibition of tyrosine phosphatases." Journal of Cell Science **115**(Pt 21): 4133-48.
Szabo, G., L. Girouard, et al. (1996). "Acute ethanol treatment augments interleukin-12 production in activated human monocytes." Annals of the New York Academy of Sciences **795**: 422-5.
Szabo, G., L. Girouard, et al. (1998). "Regulation of monocyte IL-12 production: augmentation by lymphocyte contact and acute ethanol treatment, inhibition by elevated intracellular cAMP." International Journal of Immunopharmacology **20**(9): 491-503.
Szuster-Ciesielska, A., J. Daniluk, et al. (2000). "Serum levels of cytokines in alcoholic liver cirrhosis and pancreatitis." Archivum Immunologiae et Therapiae Experimentalis **48**(4): 301-7.
Takahashi, M., K. Ogasawara, et al. (1996). "LPS induces NK1.1+ alpha beta T cells with potent cytotoxicity in the liver of mice via production of IL-12 from Kupffer cells." Journal of Immunology **156**(7): 2436-42.
Taniguchi, H., T. Toyoshima, et al. (1996). "Presence of hematopoietic stem cells in the adult liver.[see comment]." Nature Medicine **2**(2): 198-203.
Tengku-Muhammad, T. S., T. R. Hughes, et al. (2000). "Cytokine-mediated differential regulation of macrophage activator protein-1 genes." Cytokine **12**(6): 720-6.
Thompson, R. W., H. L. Valentine, et al. (2002). "In vivo and in vitro hepatotoxicity and glutathione interactions of N-methyldithiocarbamate and N,N-dimethyldithiocarbamate in the rat." Toxicological Sciences **70**(2): 269-80.
Thornton, A. J., J. Ham, et al. (1991). "Kupffer cell-derived cytokines induce the synthesis of a leukocyte chemotactic peptide, interleukin-8, in human hepatoma and primary hepatocyte cultures." Hepatology **14**(6): 1112-22.
Tirmenstein, M. A., C. X. Hu, et al. (2002). "Effects of troglitazone on HepG2 viability and mitochondrial function.[see comment]." Toxicological Sciences **69**(1): 131-8.
Tsukamoto, H., W. Horne, et al. (1995). "Experimental liver cirrhosis induced by alcohol and iron." Journal of Clinical Investigation **96**(1): 620-30.
Tsukamoto, H., S. J. Towner, et al. (1986). "Ethanol-induced liver fibrosis in rats fed high fat diet." Hepatology **6**(5): 814-22.
Tukov, F. F., J. F. Maddox, et al. (2006). "Modeling inflammation-drug interactions in vitro: a rat Kupffer cell-hepatocyte coculture system." Toxicology in Vitro **20**(8): 1488-99.
Vanhaecke, T., K. O. Lindros, et al. (2000). "Effect of ethanol on the expression of hepatic glutathione S-transferase: an in vivo/in vitro study." Biochemical Pharmacology **60**(10): 1491-6.
Wanson, J. C., D. Bernaert, et al. (1979). "Morphology and functional properties of isolated and cultured hepatocytes." Progress in Liver Diseases **6**: 1-22.
Watt, A. J., R. Zhao, et al. (2007). "Development of the mammalian liver and ventral pancreas is dependent on GATA4." BMC Developmental Biology **7**: 37.
Weinstein, S. L., M. R. Gold, et al. (1991). "Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages." Proceedings of the National Academy of Sciences of the United States of America **88**(10): 4148-52.
Weinstein, S. L., J. S. Sanghera, et al. (1992). "Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages." Journal of Biological Chemistry **267**(21): 14955-62.
Weltman, M. D., G. C. Farrell, et al. (1998). "Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis." Hepatology **27**(1): 128-33.
Wenner, C. A., M. L. Guler, et al. (1996). "Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development." Journal of Immunology **156**(4): 1442-7.

Werck-Reichhart, D. and R. Feyereisen (2000). "Cytochromes P450: a success story." Genome Biology **1**(6): REVIEWS3003.
Vidali, M., S. F. Stewart, et al. (2003). "Genetic and epigenetic factors in autoimmune reactions toward cytochrome P4502E1 in alcoholic liver disease." Hepatology **37**(2): 410-9.
Videla, L. A., V. Fernandez, et al. (2003). "Oxidative stress-mediated hepatotoxicity of iron and copper: role of Kupffer cells." BioMetals **16**(1): 103-11.
Viitala, K., Y. Israel, et al. (1997). "Serum IgA, IgG, and IgM antibodies directed against acetaldehyde-derived epitopes: relationship to liver disease severity and alcohol consumption." Hepatology **25**(6): 1418-24.
Viitala, K., K. Makkonen, et al. (2000). "Autoimmune responses against oxidant stress and acetaldehyde-derived epitopes in human alcohol consumers." Alcoholism: Clinical & Experimental Research **24**(7): 1103-9.
Wolf, K. K., S. G. Wood, et al. (2004). "Role of mouse CYP2E1 in the O-hydroxylation of p-nitrophenol: comparison of activities in hepatic microsomes from Cyp2e1(-/-) and wild-type mice." Drug Metabolism & Disposition **32**(7): 681-4.
Woodcroft, K. J., M. S. Hafner, et al. (2002). "Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression." Hepatology **35**(2): 263-73.
Woodward, J. and J. Neuberger (1997). "Sense and sensitisation: in vitro testing for hepatotoxic drug hypersensitivity.[comment]." Gut **41**(4): 573-4.
Wu, D. and A. Cederbaum (2004). "Glutathione depletion in CYP2E1-expressing liver cells induces toxicity due to the activation of p38 mitogen-activated protein kinase and reduction of nuclear factor-kappaB DNA binding activity." Molecular Pharmacology **66**(3): 749-60.
Wu, D. and A. I. Cederbaum (1999). "Ethanol-induced apoptosis to stable HepG2 cell lines expressing human cytochrome P-4502E1." Alcoholism: Clinical & Experimental Research **23**(1): 67-76.
Xu, X. R., J. Huang, et al. (2001). "Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding noncancerous liver." Proc Natl Acad Sci U S A **98**(26): 15089-94.
Yin, M., M. D. Wheeler, et al. (1999). "Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice." Gastroenterology **117**(4): 942-52.
Yokoyama, H., T. Horie, et al. (1995). "Oxidative stress in isolated rat hepatocytes during naproxen metabolism." Biochemical Pharmacology **49**(7): 991-6.
Yokoyama, H., T. Horie, et al. (2006). "Naproxen-induced oxidative stress in the isolated perfused rat liver." Chemico-Biological Interactions **160**(2): 150-8.
Yoo, J. S., F. P. Guengerich, et al. (1988). "Metabolism of N-nitrosodialkylamines by human liver microsomes." Cancer Research **48**(6): 1499-504.
Yoshimoto, T. and W. E. Paul (1994). "CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3." Journal of Experimental Medicine **179**(4): 1285-95.
Yuan, J. S., A. Reed, et al. (2006). "Statistical analysis of real-time PCR data." BMC Bioinformatics **7**: 85.
Yuan, J. S., D. Wang, et al. (2008). "Statistical methods for efficiency adjusted real-time PCR quantification." Biotechnology Journal **3**(1): 112-23.
Zaret, K. S. (1996). "Molecular genetics of early liver development." Annual Review of Physiology **58**: 231-51.
Zhou, Z., L. Wang, et al. (2003). "A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-alpha production." American Journal of Pathology **163**(3): 1137-46.
Zima, T., L. Fialova, et al. (2001). "Oxidative stress, metabolism of ethanol and alcohol-related diseases." Journal of Biomedical Science **8**(1): 59-70.