COMPLEMENT SYSTEM AND ALCOHOLIC LIVER DISEASE

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ACADEMIC DISSERTATION

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CONTENTS

I LIST OF ORIGINAL PUBLICATIONS…… 5

II ABBREVIATIONS …….. 6

III ABSTRACT ..…… 7

IV REVIEW OF THE LITERATURE ……..... 8

1. ALCOHOL-INDUCED LIVER DISEASE (ALD) …… 8
1.1. Main features of ALD ……. 8
1.2. Ethanol metabolism …… 9
1.3. Mechanisms and factors involved in ethanol-induced hepatic steatosis 9
1.4. Role of Kupffer cells in ethanol-induced liver injury ……. 13
1.4.1. Role of endotoxin in Kupffer cell activation …… 13
1.4.2. CD14 endotoxin receptor ……. 14
1.4.3. Transcriptional factors ……... 14
1.4.4. Cytokines ……. 15

2. THE COMPLEMENT SYSTEM ……..... 16
2.1. Complement activation pathways ……. 17
2.2. Classical pathway (CP) …….. 17
2.3. Alternative pathway (AP) …… 17
2.4. Lectin pathway (LP) …… 18
2.5. Terminal pathway (TP) …….. 19
2.5.1. Complement-mediated lysis of cells …… 19
2.5.2. Non-lytic effects of the complement …… 20
2.5.3. Complement and apoptosis ……. 20
2.6. Complement regulation ……. 21
2.6.1. Soluble plasma complement regulators ……. 22
2.6.2. Membrane regulators of complement ……. 24
2.7. The role of C3 in pathology ……... 25
2.7.1. C3 and inflammation ……. 26
2.7.2. C3 deficiency …….. 26
2.7.3. C3 and lipid metabolism …… 27
2.8. The role of C6 in pathology …… 28

3. COMPLEMENT SYSTEM AND THE LIVER ……. 29
3.1. Biosynthesis of plasma complement components and complement receptor expression in the liver ……. 29
3.2. Complement system and liver diseases …….. 30

V SUMMARY OF THE REVIEW ……. 32

VI AIMS OF THE STUDY ……... 33

VII MATERIALS AND METHODS 34
7.1. Animals and experimental design 34
7.2. Laboratory methods 34

VIII RESULTS AND DISCUSSION 38

8.1. COMPLEMENT ACTIVATION AND MODULATION OF COMPLEMENT GENE EXPRESSION IN THE LIVER BY CHRONIC ETHANOL UPTAKE (I, III, IV) 38
8.1.1. The effect of ethanol on complement component deposition 38
8.1.2. The effect of ethanol on complement gene expression 39

8.2. ROLE OF THE COMPLEMENT TERMINAL PATHWAY IN ALCOHOL-INDUCED LIVER INJURY (I) 41
8.2.1. Effect of complement C6 deficiency on histopathological changes and parameters of liver function in alcohol-treated rats 41
8.2.2. Effect of complement C6 deficiency on inflammatory responses in alcohol-treated rats 43

8.3. ROLE OF ALTERNATIVE COMPLEMENT PATHWAY IN ALCOHOL-INDUCED LIVER STEATOSIS (II, III) 44
8.3.1. Effect of complement C3 deficiency on histopathological changes, liver function and lipid parameters in alcohol-treated mice 44
8.3.2 Effect of complement C3 deficiency on the expression of liver genes associated with lipid metabolism in alcohol-treated mice 48

IX CONCLUDING REMARKS 50

X ACKNOWLEDGEMENTS 52

XI REFERENCES 53

I LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Bykov I, Väkevä A, Järveläinen HA, Meri S, Lindros KO.
Protective function of complement against alcohol-induced rat liver damage.
Int Immunopharmacol. 2004; 4: 1445 – 1454

II Bykov I, Junnikkala S, Pekna M, Lindros KO, Meri S.
Complement C3 contributes to ethanol-induced liver steatosis in mice.
Ann Med. 2006; 38: 280 – 286

III Bykov I, Jauhiainen M, Olkkonen V, Saarikoski S, Ehnholm C, Junnikkala S, Väkevä A, Lindros KO and Meri S.
Hepatic gene expression and lipid parameters in complement $C3^{-/-}$ mice that do not develop ethanol-induced steatosis.
J Hepatol. 2007; 46: 907 – 914

IV Bykov I, Junnikkala S, Pekna M, Lindros KO and Meri S.
Effect of chronic ethanol consumption on the expression of complement components and acute phase proteins in liver.
Clin Immunol. 2007; 124: 213 – 220

II ABBREVIATIONS

ABCA1	ATP-binding cassette transporter protein, sub-family A member 1
AdR2	adiponectin receptor 2
ADRP	adipose differentiation related protein
ALT	alanine aminotransferase
AP	alternative complement pathway
ALD	alcoholic liver disease
ApoA-I	apolipoprotein A-I
ApoA-V	apolipoprotein A-V
ApoB	apolipoprotein B
ApoH	apolipoprotein H
ApoM	apolipoprotein M
ASP	acylation-stimulating protein ($C3a_{desArg}$)
AST	aspartate aminotransferase
C	complement
C1qA	complement C1qA-chain
C1qB	complement C1qB-chain
C3	complement component C3
C4bp	C4b binding protein
C8α	C8, alpha-chain
C8β	C8, beta-chain
CD14	endotoxin receptor
CD59	protectin
CN	clusterin
CP	classical complement pathway
Crry	complement receptor related protein
DAF	decay-accelerating factor (CD55)
EP	prostaglandin E receptor
FB	complement component factor B
FA	fatty acids
FD	factor D (adipsin)
FH	factor H
FI	factor I
Ig	immunoglobulin
IL-10	interleukin-10
I/R	ischemia/reperfusion
LP	lectin complement pathway
LPS	lipopolysaccharide
MAC	membrane attack complex of complement
Masp	mannan-binding lectin associated serine protease
MBL	mannan-binding lectin
MCP	membrane cofactor protein (CD46)
MDA	malondialdehyde
mRNA	messenger RNA
NF-κB	nuclear factor kappa B
P	properdin
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PLD1	phospholipase D1
PLTP	phospholipid transfer protein
PPAR-α	peroxisome proliferator activated receptor α
ROS	reactive oxygen species
SLE	systemic lupus erythematosus
SREBP-1	sterol regulatory element binding protein 1
TCC	terminal complement complex
TG	triacylglyceride
TNF-α	tumor necrosis factor α
TNFR1	tumor necrosis factor receptor 1
TP	terminal complement pathway
VN	vitronectin

III ABSTRACT

Alcoholic liver disease (ALD) is a well recognized and growing health problem worldwide. ALD advances from fatty liver to inflammation, necrosis, fibrosis and cirrhosis. There is accumulating evidence that the innate immune system is involved in alcoholic liver injury. Within the innate and acquired immune systems, the complement system participates in inflammatory reactions and in the elimination of invading foreign, as well as endogenous apoptotic or injured cells. The present study aimed at evaluating the role of the complement system in the development of alcoholic liver injury.

First, in order to study the effects of chronic ethanol intake on the complement system, the deposition of complement components in liver and the expression of liver genes associated with complement in animals with alcohol-induced liver injury were examined. It was demonstrated that chronic alcohol exposure leads to hepatic deposition of the complement components C1, C3, C8 and C9 in the livers of rats. Liver gene expression analysis showed that ethanol up-regulated the expression of transcripts for complement factors B, C1qA, C2, C3 and clusterin. In contrast, ethanol down-regulated the expression of the complement regulators factor H, C4bp and factor D and the terminal complement components C6, C8α and C9.

Secondly, the role of the terminal complement pathway in the development of ALD was evaluated by using rats genetically deficient in the complement component C6 (*C6*$^{-/-}$). It was found that chronic ethanol feeding induced more liver pathology (steatosis and inflammatory changes) in *C6*$^{-/-}$ rats than in wild type rats. The hepatic triacylglyceride content and plasma alanine aminotransferase activity increased in *C6*$^{-/-}$ rats, supporting the histopathological findings and elevation of the plasma pro-/anti-inflammatory TNF-α/IL-10 ratio was also more marked in *C6*$^{-/-}$ rats.

Third, the role of the alternative pathway in the development of alcoholic liver steatosis was characterized by using *C3*$^{-/-}$ mice. In *C3*$^{-/-}$ mice ethanol feeding tended to reduce steatosis and had no further effect on liver triacylglyceride, liver/body weight ratio nor on liver malondialdehyde level and serum alanine aminotransferase activity. In *C3*$^{-/-}$ mice alcohol-induced liver steatosis was reduced also after an acute alcohol challenge. In both wild type and *C3*$^{-/-}$ mice ethanol markedly reduced serum cholesterol and ApoA-I levels, phospholipid transfer protein activity and hepatic mRNA levels of fatty acid binding proteins and fatty acid β-oxidation enzymes. In contrast, exclusively in *C3*$^{-/-}$ mice, ethanol treatment increased serum and liver adiponectin levels but down-regulated the expression of transcripts of lipogenic enzymes, adiponectin receptor 2 and adipose differentiation-related protein and up-regulated phospholipase D1.

In conclusion, this study has demonstrated that the complement system is involved in the development of alcohol-induced liver injury. Chronic alcohol exposure causes local complement activation and induction of mRNA expression of classical and alternative pathway components in the liver. In contrast expression of the terminal pathway components and soluble regulators were decreased. A deficient terminal complement pathway predisposes to alcoholic liver damage and promotes a pro-inflammatory cytokine response. Complement component C3 contributes to the development of alcohol-induced fatty liver and its consequences by affecting regulatory and specific transcription factors of lipid homeostasis.

IV REVIEW OF THE LITERATURE

1. ALCOHOL-INDUCED LIVER DISEASE (ALD)

1.1. Main features of ALD

Although alcoholic liver disease (ALD) is recognized as a worldwide major health problem, the pathogenetic mechanisms are still poorly understood. ALD includes fatty liver (steatosis), hepatitis, fibrosis and liver cirrhosis, which develops in 10 - 20% of alcoholics. Recent experimental animal models of ALD have also shown a wide array of alcohol-induced hepatic changes including fatty liver, hepatocellular necrosis, infiltration by inflammatory cells, hepatic venular sclerosis, and proliferation of the smooth endoplasmic reticulum (MacSween and Burt 1986; Nanji et al. 1989; Nagy 2003, Nagy 2004; Deng 2005; Tsukamoto 2005). Fatty liver (steatosis) is the earliest stage of liver injury and the most common histological finding in ALD. It occurs in response to acute or chronic alcohol intake as a result of depletion of β-oxidation of fatty acids, increase in TG synthesis and mobilization of extrahepatic free FA (Lieber 2004). Accumulation of neutral lipids in hepatocytes leads to micro- and macro-vesicular steatosis and ballooning cell degeneration (Tsukamoto 2005). Much evidence supports the notion that steatosis sensitizes the hepatocytes to more severe damage (Nanji et al. 1989). A common consequence is inflammation (steatohepatitis) associated with an increase in infiltrating granulocytes, monocytes and lymphocytes and hepatocyte death via necrosis and apoptosis (Bautista 2000; Diehl 2005). Alcohol-induced hepatic inflammation is considered a reversible condition, characterized by infiltration of polymorphonuclear leukocytes, hepatocyte degeneration and necrosis. It occurs as the most important precursor of liver cirrhosis. Hepatic fibrosis is a consequence of chronic ethanol consumption and is considered as the first irreversible stage of ALD (Worner and Lieber 1985; MacSween and Burt 1986). Hepatic fibrosis in ALD is characterized by the distortion of nomal hepatic architecture by scar tissue. The development of fibrosis and cirrhosis is a complex process that involves activation and transformation of both parenchymal cells and nonparenchymal hepatic stellate cells (HSCs) into myofibroblasts and an increased deposition of extracellular matrix in the liver (Friedman 2000). Oxygen-derived free radicals (O_2^-), inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-2), ethanol itself and its metabolite acetaldehyde are important

contributors to the activation of HSCs during liver injury (Lieber 1991; Niemelä et al. 1995; Nieto 2006; Thirunavukkarasu et al. 2006).

1.2. Ethanol metabolism

Ethanol is mainly oxidized to acetaldehyde in the liver by cytosolic alcohol dehydrogenase (ADH, EC 1.1.1.1). ADH is responsible for about 90% of ethanol oxidation. It is predominantly expressed in the liver and to a less extent in other tissues, including gastric mucosa, lungs and kidneys (Lieber 2000). Subsequent oxidation of acetaldehyde to acetate is catalyzed by aldehyde dehydrogenase (ALDH, EC 1.2.1.3). Genetic polymorphism of the ADH and ALDH genes and differential regulation at the transcriptional level contribute somewhat to ethanol elimination rate and to susceptibility to alcohol dependence and liver damage in humans (Lieber 2000; Harada et al. 2001). The microsomal ethanol oxidizing system (MEOS, cytochrome P4502E1 (CYP2E1)) which is induced in chronic alcoholics, represents a minor pathway for ethanol elimination. The CYP2E1 pathway of ethanol oxidation generates reactive oxygen species that could contribute to ethanol-induced liver injury (Lieber 2000). Reduction of NAD during ethanol and acetaldehyde oxidation causes significant changes in cellular redox potential, provides more substrate for FA synthesis and diminishes mitochondrial β-oxidation.

1.3. Mechanisms and factors involved in ethanol-induced hepatic steatosis

At the initial stage of alcohol consumption a shift in the NAD/NADH redox state of the hepatocytes results in accumulation of free FA. During ethanol oxidation the enhanced generation of NADH disrupts mitochondrial β-oxidation thereby diminishing the rate of lipid oxidation. This is one of the mechanisms of TG accumulation in the liver during chronic ethanol exposure. Specific transcription factors and genes are also involved in the regulation of lipid homeostasis during ALD development (Eaton et al. 1997; Koteish and Diehl 2001; Deaciuc et al. 2004 You and Crabb 2004 (a,b)).

SREBP-1 upregulation and increased expression of lipogenic enzymes by ethanol

In a number of recent animal models chronic ethanol exposure has been found to increase expression of genes regulating FA synthesis, suppression of genes for FA oxidation and transport from the liver have been described. Specific transcription factors including sterol regulatory element binding protein (SREBP) and peroxisomal proliferating factor α (PPAR-α) regulate the expression of many of the genes involved in FA synthesis and oxidation (Smith 2002). Sterol response element binding proteins are a family of membrane-bound transcription factors that includes three isoforms: SREBP-1a, 1c and 2. SREBP-1a and 1c regulate genes involved in hepatic TG synthesis (acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase 1, malic enzyme, and ATP citrate lyase). SREBP-2 participates in the regulation of genes required for cholesterol synthesis (HMG-CoA synthase, HMG-CoA reductase) (Horton and Shimomura 1999; Horton et al. 2002). Overexpression of liver SREBP-1a or SREBP-1c results in liver steatosis with dramatic increases in hepatic TG content. High expression of SREBP-1 was found in animals with experimental diabetes (ob/ob mouse), transgenic mice overexpressing SREBP-1c or SREBP-1a in adipose tissue, in *ICAM-1*$^{-/-}$, a transgenic mouse fed high-fat diet (Shimomura et al. 1999; Gregoire et al. 2002; Horton et al. 2003). Chronic ethanol intake induced an increased expression of SREBP-1 mRNA and protein in mouse liver (Ji and Kaplowitz 2003). An involvement of insulin-dependent mechanism in chronic ethanol-induced SREBP-1 activation has been proposed (Horton et al. 2002). It was hypothesized that hyperhomocysteine-induced endoplasmic reticulum stress during intragastric ethanol exposure may be also related to induction of SREBP-1 mRNA (Ji and Kaplowitz 2003). ERK1/2-dependent phosphorylation by ethanol can enhance the transcriptional activity of SREBP-1 and SREBP-2 (Chen et al. 1998; Lee et al. 2002). The mechanism of ethanol-induction of SREBP-1 may be also due to inhibition of the AMP-activated protein kinase (AMPK) catalyzed the phosphorylation of target enzymes, some of which are important rate-limiting enzymes in hepatic lipid metabolism. By inhibiting the activity of AMPK, ethanol relieves suppression of SREBP-1, causing its activation and increasing its levels in the nucleus (Donohue 2007).

Down-regulation of the PPAR-α transcription factor and decline in FA oxidation by ethanol

The peroxisome proliferator-activated receptor (PPAR) family of transcription factors belongs to the nuclear hormone receptor superfamily, which regulates the expression of genes involved in FA oxidation in mitochondria, peroxisomes and microsomes and possesses peroxisome proliferator response elements in their promoter regions (Lee et al. 1995; Reddy 2001). Such genes encode FA transporters (carnitine palmitoyl transferase, apolipoprotein B, the microsomal TG transfer protein, FA binding protein, and acyl-CoA dehydrogenase). In wild-type animals fasting increases the expression of PPAR-α-dependent genes regulating FA oxidation and PPAR-α-null mice develop a severe hepatic steatosis (Li et al. 2000). Chronic ethanol decreases PPAR-α mRNA expression in rats and decreases the PPAR-α DNA binding activity and transcriptional activation of PPAR-α-dependent genes in hepatoma cells or primary rat hepatocytes as reflected by a reduced binding by the RXR-PPAR-α complex to a PPAR-α-specific promoter sequence (Wan et al. 1995; Galli et al. 2001). This effect of ethanol is enhanced by using cyanamide, an ALDH inhibitor, and is abolished by the ADH inhibitor, 4-methylpyrazole. Decreased PPAR-α binding was associated with decreased expression of PPAR-α-dependent genes (medium chain acyl-CoA dehydrogenase) (Fischer et al. 2003). PPAR-α agonist (WY14, 643) restored PPAR-α DNA binding activity and prevented the development of chronic ethanol-induced steatosis in mice (Fischer et al. 2003). Induction of PPAR-α by agonist in these animals blocked fatty liver development by accelerating FA oxidation.

The role of adiponectin in alcohol-induced liver steatosis

In addition to transcriptional factors, the regulatory factor adiponectin is involved in regulation of lipid metabolism. Adiponectin is a tissue-derived (mainly adipocyte-derived) serum molecule (also called ACRP30 and GBP28), which is characterized by anti-inflammatory, antidiabetic and antiatherogenic properties. Adiponectin shares sequence homology with a family of proteins that show a characteristic N-terminal collagen-like region, a C-terminal, complement factor C1q-like globular domain and a TNF-α-like three-dimensional structure (Shapiro and Scherer 1998). Insulin resistance, obesity, type 2 diabetes mellitus and coronary artery disease are associated with decreased circulating adiponectin levels (Guo et al. 2007).

Adiponectin decreases hepatic TG deposition by reducing free FA plasma level and their hepatic influx (Bajaj et al. 2004; Lopez-Bermejo et al. 2004; Matsuzawa et al. 2004). Adiponectin has an anti-inflammatory and antifibrotic effect in experimental murine models of liver damage, in non-alcohol fatty liver disease (NAFLD) and alcoholic fatty liver and in LPS-induced liver injury (Kamada et al. 2003; Xu et al. 2003; Masaki et al. 2004). Chronic ethanol administration causes a significant reduction in circulating adiponectin, which is associated with ethanol-elicited reduction in FA oxidation. The condition can be restored by treatment with recombinant adiponectin (Xu et al. 2003). Moreover, such treatment decreases the levels of TNF-α, because adiponectin and TNF-α are known to regulate each other's production. Adiponectin and TNF-*α* seem to have opposite effects on lipid metabolism and inflammation. Adiponectin antagonises both production and activity of TNF-*α* and TNF-*α* has been shown to inhibit adiponectin production (Diehl 2005). An imbalance in the production of adiponectin and TNF-*α* promotes damage in many tissues, including the liver (Hui et al. 2004). Accumulating evidence now suggests that hepato-protective activity of adiponectin is due, at least in part, to a direct anti-inflammatory effect of adiponectin on Kupffer cells (Park et al. 2006).

ADRP in liver steatosis

Adipose differentiation-related protein (ADRP; also known as ADFP or adipophilin) is actively involved in hepatic lipid metabolism. ADRP is abundantly expressed in a variety of cells, including hepatocytes, macrophages, breast epithelium, and kidneys, in which ADRP coats lipid droplets (LD) (Brasaemle et al. 1997; Heid et al. 1998). ADRP shares sequence homology with other LD proteins including perilipin, Tip47 and S3-12. ADRP activates long-chain FA uptake and TG formation, while suppression of ADRP decreases lipid accumulation in hepatocytes (Gao and Serrero 1999; Imamura et al. 2002; Larigauderie et al. 2004; Schadinger et al. 2005). ADRP has been shown to increase in fatty liver in humans and rodents (Dalen et al. 2006; Motomura et al. 2006). It has been demonstrated that ADRP antisense oligonucleotide (ASO) treatment specifically decreases ADRP mRNA and protein levels in the livers of $Lep^{ob/ob}$ mice and diet-induced obese mice. This resulted in suppression of lipogenic genes, reduction in TG secretion, reversal of hepatic steatosis and hypertriglyceridemia, and enhancement of insulin sensitivity (Imai et al. 2007). Increased expression of ADRP was associated with FA accumulation in

hepatocytes in patients with nonalcoholic fatty liver disease with abnormal liver dysfunction (Kohjima et al. 2007). ADRP deficiency in mice caused reduction in hepatic TG and resistance to diet-induced fatty liver (Chang et al. 2006).

1.4. Role of Kupffer cells in ethanol-induced liver injury

The innate immune system plays a significant role in the first line of defense against invading pathogens. Macrophages and neutrophils (phagocytic cells) participate in the response against invading pathogens. Experimental data suggest that Kupffer cells, the liver resident macrophages, play a key role in the pathogenesis of alcohol-induced liver injury. For instance, Kupffer cell depletion by gadolinium chloride increases fatty infiltration, inflammation and necrosis as well as serum levels of AST and ALT and free radicals after chronic ethanol treatment (Wheeler et al. 2001; Hines and Wheeler 2004).

1.4.1. Role of endotoxin in Kupffer cell activation

Endotoxin (lipopolysaccharide; LPS) has been shown to activate Kupffer cells after ethanol consumption (Nagy 2003; You and Crabb 2004 (a,b)). LPS is a component of the outer membrane of intestinal gram-negative bacteria (Han 2002; Thakur et al. 2007). Gut-derived LPS in the portal blood may promote hepatocellular injury (Schafer et al. 2002). Moreover, sterilization of the gut with nonabsorbable antibiotics, which kill gram-negative bacteria, blocks alcohol-induced liver injury (Adachi et al. 1995). Alcohol-induced increased gut permeability to endotoxin is considered to be one of the initial mechanisms by which ethanol increases Kupffer cell activation and subsequent cytokine production. Proinflammatory cytokines and chemokines released by Kupffer cells in response to LPS stimulation exert a range of autocrine and paracrine effects that initiate defense responses and promote infiltration of inflammatory cells and induced oxidative responses in the liver (Nanji et al. 1993; Thurman 1998; Enomoto et al. 2000; Bode and Bode 2003). Ethanol is known to alter cytokine levels and mRNA expression in a variety of tissues including blood, lung, liver, and brain. Proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, are

released from Kupffer cells or infiltrating neutrophils and macrophages and elicit defensive responses in parenchymal cells, including activation of apoptosis often seen in alcoholics and animals treated with ethanol. A significant role for TNF-α in ethanol-induced hepatotoxicity has been indicated both by clinical observations and in animal studies (McClain et al. 1999; Tilg and Diehl 2000). In support, treatment with neutralizing antibodies to TNF-α and depletion of Kupffer cells has been found to suppress ethanol-induced liver injury (Adachi et al. 1994; Iimuro et al. 1997).

1.4.2. CD14 endotoxin receptor

It is well known that LPS bound to LPS-binding protein (LBP) interacts with the CD14 endotoxin receptor, a glycosyl-phosphatidylinositol-anchored membrane protein, found on mononuclear cells, including Kupffer cells. To initiate an intracellular responce CD14 requires an association with Toll-like receptors (TLR) because CD14 does not have a transmembrane-spanning domain. CD14 and TLR appear to be involved in the development of alcohol-induced liver injury. Experimental chronic ethanol exposure has been found to upregulate the expression of CD14 mRNA and protein, and CD14- and LBP-deficient mouse strains are resistant to alcohol-induced liver injury (Su et al. 1998; Lukkari et al. 1999; Uesugi et al. 2002). An association between human ALD and polymorphisms in the promoter of the CD14 gene has been described (Järveläinen et al. 2001). Chronic ethanol intake also sensitizes Kupffer cells to LPS-mediated activation by increasing the ability of LPS to stimulate members of the mitogen-activated protein kinase family, ERK1/2 and p38 (Järveläinen et al. 1997; Kishore et al. 2001; Cao et al. 2002; Kishore et al. 2002; Koteish et al. 2002).

1.4.3. Transcriptional factors

It is widely accepted that also reactive oxygen species (ROS) play a significant role in the development of ALD probably in Kupffer cell activation. ROS participate in the activation of NF-κB and may have an indirect effect on signaling pathways and on the ubiquitin-proteosomal pathway for protein degradation (Hoek et al. 1992; Hoek and Pastorino 2002). Inhibition of NF-κB activity in the liver via transduction with the IκB super-repressor prevents alcohol-induced liver injury. This demonstrates the functional significance of NF-κB activation in the pathogenesis of ALD (Uesugi et al.

2001). Activation of activator protein 1 (AP-1) by ethanol can also be important in mediating the inflammatory phase of ethanol-induced liver injury. AP-1 regulates the transcription of genes involved in inflammation and fibrosis, including TNF-α, CD14, matrix metalloproteinases and collagen type I (Armendariz-Borunda et al. 1994; Tsai et al. 2000; Wheeler and Thurman 2003).

1.4.4. Cytokines

TNF-α is one of the principal mediators of the inflammatory response in mammals. It provides transducing differential signals that regulate cellular activation and proliferation, cytotoxicity and apoptosis. On hepatocytes TNF-α acts through two types of plasma membrane receptors: p55 [type 1 tumor necrosis factor receptor (TNFR1)] and p75 [type 2 tumor necrosis factor receptor (TNFR2)] (Yin et al. 1999). Binding of TNF-α to TNFR1 leads to activation of the apoptotic machinery or to cytoprotective responses in hepatocytes.

Increased serum TNF-α levels in patients with ALD have been found to correlate with mortality, supporting the idea that TNF-α may be important in ALD. Moreover, mice deficient in TNFR1 exhibited essentially no pathological changes in liver and plasma after chronic ethanol treatment. Kupffer cells also produce large amounts of potentially damaging free radicals including superoxide via phagocytic NADPH oxidase system. It has been shown that mice deficient in $p47^{phox}$, a regulatory subunit of the oxidase, after 4 wk of enteral ethanol feeding, had significantly reduced liver injury and blunted cytokine production indicating a link between oxidant production and cytokine generation in this model of ALD (Kono et al. 2000). Suppression of inflammatory gene expression, inhibition of neutrophil recruitment, removal of inflammatory cells by apoptosis and phagocytosis and induction of anti-inflammatory mediators are involved in the resolution of an inflammatory response in alcohol liver injury (Gregory and Wing 2002). The balance between both pro- and anti-inflammatory cytokines seems to be important in order to assess the nature of inflammatory response (Latvala et al. 2005). IL-10, glucocorticoids, cAMP, and prostaglandins are important anti-inflammatory mediators. Chronic ethanol consumption causes an enhanced expression of the most potent anti-inflammatory cytokine IL-10 in rodents (Koteish et al. 2002; Lawrence et al. 2002). IL-10 is known as a cytokine synthesis inhibiting factor and represents one of the most important

immune-regulating cytokines. IL-10 has multiple biological effects on different cell types. It is produced by macrophages, T cells, B cells, mast cells, keratinocytes, and some tumor cell lines (Moore et al. 1993; Moore et al. 2001). The effect of IL-10 on immune responses is mostly inhibitory on T cells. It suppresses macrophage production of cytokines and chemokines (McInnes et al. 2001; Denys et al. 2002). It has been shown, that IL-10 may protect against hepatotoxicity in several models of experimental liver injury including acetaminophen-, concanavalin A- and LPS-induced liver injury (Emoto et al. 2003; Bourdi et al. 2007; Erhardt et al. 2007).

2. THE COMPLEMENT SYSTEM

Complement is a central part of the innate immune system. It plays an important role in defense against invading pathogens and in the clearance of cellular debris. Complement has multiple functions including opsonization of pathogens and debris by C3b, regulation of inflammation via anaphylatoxic fragments C5a and C3a and in providing a link between innate and adaptive immunity through C3 receptors on B cells and antigen-presenting cells. The complement system consists of about 40 different proteins found in blood, other body fluids as well as cells and tissues.

C1q, C4b, C3b, and iC3b are ligands for complement receptor 1 (CR1) and complement receptor 3 (CR3). Phagocytosis is mediated by receptors CR1 and CR3, which are localized e.g. on neutrophils and antigen presenting cells such as macrophages.

Complement-mediated inflammation is generated primarily by the activation products C3a and C5a. They increase vascular permeability and contraction of smooth muscle cells. C3a and C5a act also as chemotactic homing signals for leukocytes to sites of inflammation. By binding to specific receptors (C5aR and C3aR), C5a and to a lesser extent also C3a, mediate anaphylaxis e.g. by releasing histamine from mast cells. Membrane attack complex of complement (MAC) itself can also activate cells via Ca^{2+} influx to release leukotrienes, prostaglandins and other inflammatory mediators to induce further inflammatory changes in tissues.

Complement also participates in the clearance of necrotic and apoptotic cells from damaged or regenerating tissues. This occurs via opsonization of immune complexes

and cellular debris by C3b and C4b. C1q also recognizes apoptotic cells or blebs directly resulting in opsonization and phagocytosis. A deficiency or failure in this system leads to systemic lupus erythematosus with characteristic symptoms. Complement system is also involved in certain pathological conditions such as different forms of nephropathy and ischemia/reperfusion injury.

2.1. Complement activation pathways

Complement activation occurs via three different pathways: the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP) (Fig 1). In the final common step a protein complex, the MAC on a complement-activating surface is formed.

2.2. Classical pathway (CP)

The CP is activated when its recognition molecule C1q binds to immunoglobulins (IgG, IgM), certain acute phase protein (CRP, SAP, PTX3), charged molecules, apoptotic or necrotic cell debris (Mold et al. 1999; Walport 2001 (a,b)). Binding to surface induces a conformational change in the C1q and results in the activation of the associated molecules C1r and C1s. C4b2a complex is formed by the C1s-induced activation of C4 and C2, and further activates the central complement component C3.

2.3. Alternative pathway (AP)

Activation of the antibody-independent AP is due to C3(H_2O), which is continuously formed by spontaneous hydrolysis of an internal thioester in C3 (Pangburn and Müller-Eberhard 1986; Thurman and Holers 2006; Zipfel et al. 2007). Activated C3 binds factor B, whereafter factor B is cleaved and activated by factor D and C3(H_20)Bb is formed. This complex is stabilized by properdin and can subsequently activate further C3 molecules to C3b. C3b can bind factor B and the subsequent

activation of factor B leads to the formation of a C3bBb convertase and cleavage of further C3. Factor H and I control the activation of the alternative pathway by degrading C3b.

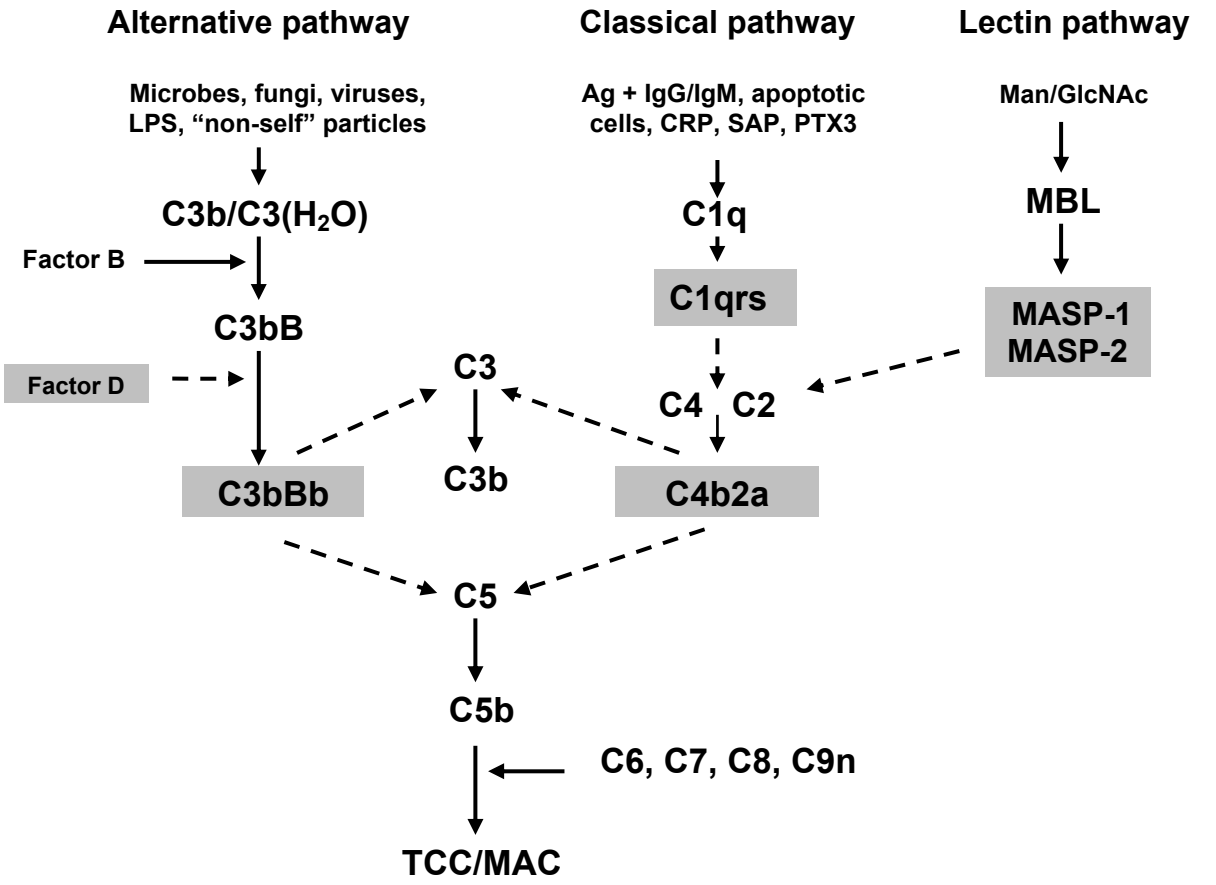

Figure 1. The complement activation cascades. The dashed lines indicate enzymatic activities, brickets – active enzyme or enzyme complex. CRP – C reactive protein, LPS – lipopolysaccharide, SAP - serum amyloid P component, PTX3 – pentraxin 3, Man – mannose, GlcNAc – N-acetyl glucosamine, MBL - mannan-binding lectin, MASP - mannan-binding lectin associated serine protease, TCC/MAC - terminal complement complex/membrane attack complex of complement.

2.4. Lectin pathway (LP)

Initiation of the LP occurs when plasma mannan-binding lectin (MBL) binds directly to mannose or *N*-acetyl glucosamine residues present abundantly on target cells e.g. yeast. MBL has a structure similar to that of C1q and ficolins (L-ficolin and H-ficolin). After binding it activates MBL-associated serine proteases (MASPs-1 to -3) (Turner 1996; Schwaeble et al. 2002). Only MASP-2 has a role in complement activation,

corresponding to that of C1s in the CP (Thiel et al. 1997; Rossi et al. 2001). MASP-2 cleaves C4 to produce the C4a and C4b fragments and C2 to generate C2b and C2a. C4b and C2a together form the C3 convertase (C4b2a), the enzyme that catalyses the next step in the reaction pathway (Kerr 1980).

2.5. Terminal pathway (TP)

Activation of complement leads to the TP, which is the same for all three initiating pathways. C5 is cleaved by either the classical or the alternative C5 convertase and subsequent binding of C6, C7, C8, and C9 results in the formation of the terminal complement complex (TCC) The membrane associated form is called the membrane attack complex (MAC). C5b binds C6 to form the stable bimolecular complex C5b6. If C7 availability is limited, the stable bimolecular C5b6 complex remains in solution. In the presence of C7 fluid phase C5b-7 is formed. This form has an ability to attach to target membranes (Thompson and Lachmann 1970). Membrane-bound and fluid-phase C5b-7 are capable of binding C8. C5b-8 initiates polymerization of C9 by binding C9 via C8. Formation of the C5b-9 complex results in water permeable hydrophilic pores which are inserted in the phospholipid bilayer of the membranes. Finally, MAC causes rupture of the cell membranes of a susceptible target cell. It has been suggested that the complement system plays a role as a physiological waste-disposal mechanism, particularly in the clearance of dying cells and immune complexes and that complement deficiency impairs normal mechanisms of waste disposal and, consequently, this material can be a source of autoantigens (Botto and Walport 1994). On host nucleated cells, complement activation is often sublytic, which offers some protection to the cell, when they activate their defense systems (Morgan 1989 (a,b)).

2.5.1. Complement–mediated lysis of cells

MAC formation is critical for complement-mediated lysis of target cells. Insertion of C9 through the cell lipid bilayer membranes and polymerization results in the formation of water permeable hydrophilic pores and subsequent rupture of the cell membranes. The efficiency of the lysis depends on the number of MACs on the cell

membrane (Cole and Morgan 2003). Erythrocytes are much more sensitive to the lytic effects of complement compared to nucleated cells. The latter are better protected because of a variety mechanisms including ion pumps, mechanisms for removal of the MAC and a more abundant presence of membrane-bound complement regulators (Morgan 1989 (a)). MACs can be removed by shedding of membrane vesicles (ectocytosis) or by internalization and degradation (Carney et al. 1985; Morgan et al. 1987; Scolding et al. 1989).

2.5.2. Non-lytic effects of the complement

MAC also affects the inflammatory response by activating neutrophils and macrophages to produce and release inflammatory mediators (prostaglandins, thromboxanes, leukotrienes and reactive oxygen species) (Hansch et al. 1984). Several signal transduction pathways are initiated by Ca^{2+} intake and the activation of receptor tyrosine kinases, G-proteins, MAP kinase, NF-kB and c-Jun N-terminal kinase pathways (Cybulsky et al. 1990; Cybulsky et al. 1999; Rus et al. 2001; Takano et al. 2001; Peng et al. 2002). A role for non-lytic activating effects of the MAC has been described in glomerulonephritis, dilated cardiomyopathy vascular smooth muscle proliferation and remodelling in atherosclerosis (Niculescu and Russ 1999). MAC has also been implicated as a stimulus to cell proliferation in models of mesangioproliferative glomerulonephritis, diabetes (retinopathy and nephropathy), atherosclerosis and production of platelet-derived (PDGF) and basic fibroblast growth factors (bFGF) (Benzaquen et al. 1994; Brandt et al. 1996; Niculescu et al. 1999). Sublytic MAC can directly stimulate proliferation of different kind of cells like aortic smooth muscle cells and Schwann cells (Niculescu et al. 1999; Dashiell et al. 2000).

2.5.3. Complement and apoptosis

Complement plays significant roles in regulating apoptosis, which is important in development and homeostasis, embryogenesis and tissue remodeling (Nauta et al. 2003). Several different products of complement including anaphylatoxins participate in this process. C5a inhibits spontaneous apoptosis of neutrophils and protects neurons from glutamate neurotoxicity, probably via inhibition of caspase 3 activity

(Lee et al. 1993; Pitt et al. 2000; Mukherjee and Pasinetti 2001). C3a has also been shown to protect neurons from apoptosis induced by *N*-methyl-D-aspartate (van Beek et al. 2001). Non-lytic MAC also modulates apoptosis by promoting the survival of oligodendrocytes, increasing Bcl-2 transcription and suppressing the activation of caspase 3. It also up-regulates anti-apoptotic genes and down-regulates pro-apoptotic genes in the brain as shown in the rodent multiple sclerosis model (Rus et al. 2006). Complement has been implicated in cell apoptosis in ischaemia/reperfusion injury, myocardial infarction and stroke models (D'Ambrosio et al. 2001; Monsinjon et al. 2001). Blocking the generation of MAC and C5a by anti-C5 antibodies attenuated both lysis and apoptosis of myocardial cells (Daemen et al. 1999). Complement participates in the clearance of apoptotic cells by helping professional phagocytes and to prevent an inflammatory response (Fadok et al. 1998; Savill and Fadok 2000). C1q and mannan-binding lectin, can bind blebs that initiate activation of the CP and LP and deposition of other complement fragments (Korb and Ahearn 1997). C1q binds specific receptors on the phagocyte surface, including CD91 and calreticulin to recruit phagocytes and initiate phagocytosis. C3 fragments deposited on the apoptotic cells bind to receptors CR1, CR3 and CR4 on the phagocytes to mediate recognition and clearance (Takizawa et al. 1996; Ogden et al. 2001). Defects in the removal of apoptotic cells can induce autoimmune pathology, like SLE, supporting the so called “waste disposal” hypothesis (Walport 2001 (b)). Persisting apoptotic cells or their fragments may generate an autoimmune response because cytoplasmic and nuclear antigens become exposed at the cell surface or outside the lysed cell and may provide signals to T cells and antigen-presenting cells, which stimulate autoreactive B cells to differentiate into plasma cells and start producing autoantibodies (Mohan et al. 1993; Savill and Fadok 2000; Fishelson et al. 2001).

2.6. Complement regulation

By regulating the complement system host cells can inhibit activation of complement occurring in vivo. There are 10 plasma and membrane-bound proteins that regulate complement activation and prevent against complement attack host cells (Table 1, Fig 2).

2.6.1. Soluble plasma complement regulators

Soluble plasma complement regulators prevent excessive complement activation in the fluid phase.

C1 inhibitor (C1INH)

C1INH is an inhibitor of the CP (Cicardi et al. 2005; Wagenaar-Bos and Hack 2006). It inhibits C1r and C1s serine esterases by irreversible covalent binding. A deficiency in C1INH causes hereditary angioedema, a disease characterized by attacks of edema and pain (Frank 2005). In addition C1INH also inhibits MASP1 and MASP2 to prevent LP activation.

Table 1. Complement regulators

Protein	Function
Soluble regulators	
C1 inhibitor (C1INH)	Inhibitor of C1r and C1s serine esterases and MASP1 and MASP2
C4b-binding protein (C4bp)	Accelerates decay of C4b2a, cofactor for cleavage of C4b by FI
Factor I	Cleaves C3b and C4b
Factor H	Decay acceleration activity for the C3/C5 convertase of AP, cofactor for FI
Vitronectin	Inhibits C5b-7 complex insertion into membrane
Clusterin	Inhibits C5b-7 complex insertion into membrane
Membrane regulators	
Membrane cofactor protein (MCP; CD46)	Cofactor for factor I-mediated cleavage of C3b and C4b
Decay accelerating factor (DAF; CD55)	Decay acceleration activity for the C3/C5 convertases
Protectin (CD59)	Inhibition of MAC formation
Complement receptor 1 (CR1)	Decay acceleration activity for the C3/C5 convertases, cofactor for factor I, immune complex processing and clearance

C4b-binding protein (C4bp)

C4bp accelerates the decay of the CP C3-convertase C4b2a by promoting dissociation of C2a. It can also function as a cofactor in the cleavage of C4b by FI (Blom et al. 2004).

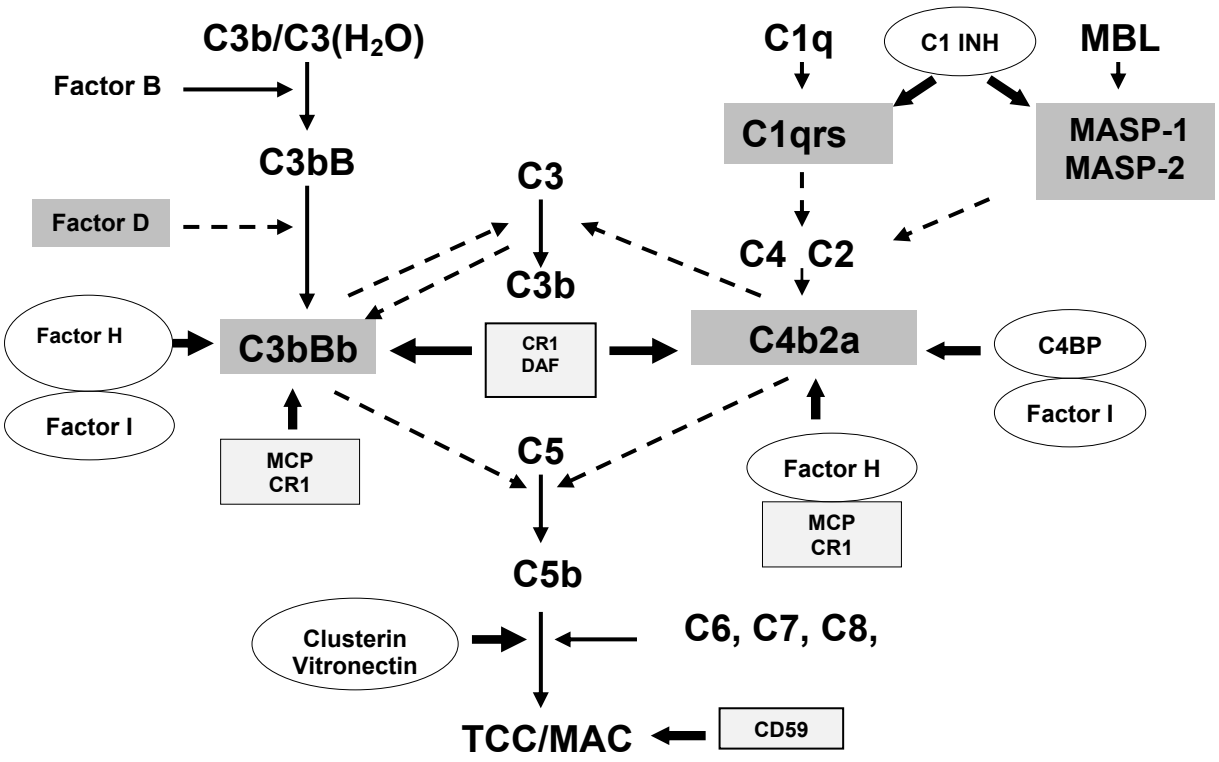

Figure 2. Regulation of complement activation. Soluble complement regulators are in the ovals, membrane-bound complement regulators are in the brickets. Thick arrows indicate an inhibitory function.

Factor H

Factor H is an essential regulatory protein that plays a critical role in the homeostasis of the complement system in plasma and in the protection of bystander host cells and tissues from damage by complement activation (Atkinson and Goodship 2007). Factor H is a glycoprotein (155 kDa) with a single polypeptide chain. The secreted form of the protein is composed of 20 repetitive units of 60 amino acids called short consensus repeats (SCR) (Nilsson and Müeller-Eberhard 1965; Ripoche et al. 1988). Factor H accelerates the decay of the alternative pathway C3-convertase (C3bBb) by binding C3b and acting as a cofactor for the factor I-mediated proteolytic inactivation

of C3b (Weiler et al. 1976; Whaley and Ruddy 1976; Pangburn et al. 1977). In the presence of factor H, factor I cleaves the α′-chain of C3b at two nearby sites generating two fragments of 68 and 43 kDa, respectively. The inactivated C3b surface-bound molecule, named iC3b, remains covalently linked to the activating surface.

Factor I

Factor I is a serine esterase that prevents the formation of the AP and CP C3 and C5 convertases by cleaving C3b and C4b to iC3b, C3c, C3dg and C4c, C4d, respectively (Sim 1993; Sim and Laich 2000).

Vitronectin and clusterin

Vitronectin and clusterin interact with the terminal complexes (C5b-7, C5b-8 and C5b-9) of complement and inhibit their insertion into lipid membranes (McDonald and Nelsestuen 1997; Preissner and Seiffert 1998).

2.6.2. Membrane regulators of complement

Complement regulatory proteins on cell membranes protect host tissues from injury, when complement is activated (Miwa and Song 2001). The regulatory membrane proteins anchored on cell surfaces are complement receptor 1 (CR1; CD35), decay-accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), and CD59. With the exception CD59 they belong to a family of proteins (regulators of complement activation, RCA) encoded by a gene cluster on chromosome 1. There are differences in the composition of membrane complement regulatory proteins between man and mouse. DAF and CD59 genes are duplicated in the mouse (daf-1, daf-2, cd59a and cd59b) (Spicer et al. 1995; Song et al. 1996; Powell et al. 1997; Qian et al. 2000). Daf-2 and cd59b are expressed only in the mouse testis whereas daf-1 and cd59a are expressed broadly. In man MCP is expressed on all cells except erythrocytes whereas in the mouse MCP expression is restricted to the testis (Cole et al. 1985; Nickells and Atkinson 1990; Liszewski et al. 1991; Johnstone et al. 1993; Miwa et al. 1998). In the mouse Crry (complement-receptor 1-related gene/protein y)

possesses both MCP and DAF activities (Holers et al. 1992; Li et al. 1993; Paul et al. 1989; Song 2004).

Membrane cofactor protein (MCP; CD46)

MCP is regulator of C3 activation by functioning as a membrane associated cofactor protein for factor I-mediated cleavage of C3b to inactive iC3b and of C4b to iC4b (Liszewski et al. 1991). MCP associates with the plasma membrane via its C-terminal transmembrane domain and an intracellular cytoplasmic tail.

Decay accelerating factor (DAF; CD55)

DAF inhibits the activation of C3 and C5 by preventing the formation of new and accelerating the decay of preformed C3 and C5 convertases (Lublin and Atkinson 1989). DAF is anchored to cell membranes via a glycosylphosphatidylinositol anchor.

Protectin (CD59)

CD59 regulates TP on cell membranes by preventing formation of the final assembly of the membrane C5b-9 complex. It binds to C8 and C9 molecules and blocks their insertion into cell membranes (Meri et al. 1990). Protectin is anchored to cell membranes via glycosylphosphatidylinositol anchor.

Complement receptor 1 (CR1)

CR1 has both DAF and MCP activities and functions as a receptor for C3b and C4b. CR1 is a major immune adherence receptor and plays a role in immune complex processing and clearance (Ahearn and Fearon 1989). CR1 associates with the plasma membrane via its C-terminal transmembrane domain.

2.7. The role of C3 in pathology

By participating in inflammatory reactions and elimination of invading foreign, apoptotic or injured cells, the complement system can play a significant role in the pathogenesis of different diseases.

2.7.1. C3 and inflammation

Complement activation leads to the generation of inflammation-inducing anaphylatoxins (complement activation products C3a, C4a and C5a). C3a participates in the process of liver regeneration and Kupffer cell activation (Puschel et al. 1993; Schieferdecker et al. 1997; Strey et al. 2003). C3 plays a central role in all three activation pathways participating in the formation of C3- and C5-convertases, production of opsonins for phagocytosis through generating C3b and iC3b, which are ligands for complement receptors CR1, CR3 and CR4 present on phagocytic cells (Lambris 1988). C3a and C5a binding to their receptors (C3aR and C5aR) leads to the degranulation of mast cells and basophils to release vasoactive mediators and cause an inflammatory reaction. C3 participates in the solubilization and clearance of immune complexes and in the clearance of apoptotic cells. iC3b bound to dead cells or cell debris is recognized by CR3 and CR4 receptors on the macrophage surface (Davies et al. 1994; Mevorach et al. 1998).

2.7.2. C3 deficiency

In humans a primary (inherited) C3 deficiency is associated with recurrent bacterial infections, mainly caused by gram-positive bacteria, usually pneumococci. Clinical manifestation of C3 deficiency includes infections in the upper and lower respiratory tracts (pneumonia, sinusitis, tonsillitis, otitis or meningitis), renal impairment (membranoproliferative glomerulonephritis) or autoimmune diseases (Imai et al. 1991; Homann et al. 1997). Acquired C3 deficiency is observed in patients with impairment in the regulatory proteins factor I or factor H. Amplification of C3 cleavage by an unregulated C3bBb C3-convertase results in *S. pneumoniae* and *N. meningitidis* infections or in diseases caused by excessive complement activation in blood vessels or kidneys (SLE, hemolytic uremic syndrome, membranoproliferative glomerulonephritis).

In animals, like guinea-pigs, partial C3-deficiency induced decreased bactericidal activity and an abnormal antibody responses to T-cell-dependent antigens (Auerbach et al. 1990). C3-deficient dogs suffered from severe bacterial infections and major renal pathology (Ameratunga et al. 1998). The use of cobra venom factor (CVF) for

depleting complement proteins induced lower amounts of specific antibodies against sheep red blood cells, impaired production of specific antibodies against another T-dependent antigen (phage φX174) and enhanced susceptibility to group B streptococcal infection and defective opsonization (Wessels et al. 1995). In pigs with factor H deficiency membranoproliferative glomerulonephritis type II was associated with excessive complement activation shown by low plasma C3, high plasma TCC and complement deposition in the renal glomeruli (Høgåsen et al. 1995). The use of C3-deficient mice (*C3*$^{-/-}$) or inhibition of complement activation with the C3 convertase inhibitor Crry-Ig prevented anti-phospholipid syndrome, fetal loss and growth retardation (Thurman et al. 2005).

2.7.3. C3 and lipid metabolism

Recent studies demonstrate a strong association between complement and lipid metabolism. Activation of the CP or AP leads to cleavage of the complement component C3 to the pro-inflammatory C3a fragment. C3a can be cleaved to $C3a_{desArg}$ by carboxypeptidase N or R (Campbell et al. 2001). $C3a_{desArg}$ has also been referred to as acylation-stimulating protein, ASP (Baldo et al. 1993). Both ASP and its precursor C3a have been suggested to be involved in the regulation of FA uptake to adipose tissue and body lipid homeostasis (Cianflone et al. 2003). Experimental data suggest that ASP stimulates TG synthesis in adipocytes and intracellular uptake of glucose by adipocytes. It increases the activity of diacylglycerol acyltransferase, stimulates reesterification of free FAs into TG in adipocytes and reduces endogenous FA production by inhibiting a hormone-sensitive lipase (Faraj et al. 2004). These results have been obtained from experiments with *C3*$^{-/-}$ mice, which cannot generate ASP and manifest various metabolic alterations in their lipid metabolism. *C3*$^{-/-}$ mice crossed with mice deficient in both apolipoprotein E and low-density lipoprotein receptor (ApoE-/- LDLR-/-) exhibited increased serum TG concentrations and a more proatherogenic lipoprotein profile with more LDL cholesterol and VLDL TG, than control mice (Persson et al. 2004). The *C3*$^{-/-}$ mice showed also a delayed postprandial TG clearance, an effect that is normalized by administration of ASP (Murray et al. 1999). In patients with nonalcoholic fatty liver disease (NAFLD) high plasma ASP and C3 levels were associated with liver steatosis (Yesilova et al. 2005).

2.8. The role of C6 in pathology

Full activation of the complement leads to the assembly of the lytic MAC, which consists of the terminal components C5b, C6, C7, C8 and multiple C9 molecules. Loss of ability to protect cells can lead to “self-damage” when complement is activated, targets are opsonized, excessive inflammation-inducing anaphylatoxins (complement fragments C3a, C4a and C5a) are generated and the MAC forms on cell surfaces (Straatsburg et al. 2000). The TP has been implicated in a large number of diseases and plays a crucial role in various pathological conditions (Niculescu et al. 1999; Niculescu and Rus 1999; Walport 2001 (a, b); McGeer and McGeer 2002). Deficiencies of the TP predispose to meningococcal infections, indicating that its cytolytic properties are of particular importance in host defense against *Neisseria* (Lehner et al. 1992; Würzner et al. 1992).

Complement component C6 plays an essential role in the formation of a stable C5b-9 terminal complement complex (Esser 1994). A number of experimental disease models have been studied in the PVG/C6^{-} rat characterized by deficiency in C6 and loss of the ability to mediate complement-dependent lysis. These animals have been shown to lack both antigenic and functional C6 due to a 31-bp deletion in exon 10 of the C6 gene (van Dixhoorn et al. 1997). It has been shown that in models of inflammatory glomerulonephritis PVG/C6^{-} rats had less proteinuria and apoptosis compared with normal PVG/c rats and were also protected from thrombotic microangiopathic glomerulonephritis, hyperacute rejection of xenografts and experimental autoimmune encephalomyelitis (Brauer et al. 1993; Nangaku et al; 1997; Sato et al. 1999; Tran et al. 2002). Studies have suggested that C6 may be important in atherosclerotic lesion progression. When C6-deficient rats were fed a cholesterol-rich diet for 14 week a protective effect of C6 deficiency on the development of diet-induced atherosclerosis was seen (Schmiedt et al. 1998).

3. COMPLEMENT SYSTEM AND THE LIVER

3.1. Biosynthesis of plasma complement components and complement receptor expression in the liver

Most complement components are produced mainly in the liver (CP: C1r/s, C2, C4, C4bp; AP: C3, factor B; LP: MBL, MASP1-3; TP: C5, C6, C8, C9; and soluble regulators (factors I, H, C1 inhibitor)) (Morris et al. 1982; Morgan and Gasque 1997; Schwaeble et al. 2002). C1q is produced by epithelial cells, fibroblasts, monocytes and macrophages (Tenner and Volkin 1986). Factor D is mainly produced in adipocytes while properdin and C7 are synthesized by macrophages and monocytes (Choy et al. 1992; Maves and Weiler 1993; Wurzner et al. 1994). Membrane bound complement components – CD59, CD35, CD46, and CD55 are produced by nearly all cell types (Mead et al. 1999; Harris et al. 1999; Qin X et al. 2001). Hepatocyte nuclear factors (HNF1, HNF3 and HNF4) as well as proinflammatory cytokines (IL-6, IL-1, TNF-α, INF-γ) control transcription of the complement components (Garnier et al. 1996; Phillips et al. 1996; Pontoglio et al. 2001; Stapp et al. 2005).

Several complement receptors are expressed in the liver. Acute phase response and glucose release can be detected in hepatocytes after LPS challenge or partial hepatectomy (Schieferdecker et al. 2001; Koleva et al. 2002). In Kupffer cells complement receptors CR1 (C3b-receptor, CD35), CR3 (iC3b- and β-glucan-receptor CD11/CD18) and CR4 (iC3b-receptor, CD11c/CD18) are expressed at high levels and participate in the clearance of C3-opsonized immune complexes from blood (Schieferdecker et al. 1997; Yan et al. 2000). Stimulation via C5aR leads to hepatocyte proliferation. C5aR can also be detected on Kupffer and stellate cells and plays an important role in the release of prostanoids, proinflammatory cytokine regulation and in the expression of fibronectin (Schieferdecker et al. 2001; Schlaf et al. 2004). Thus the liver produces the bulk of plasma complement components and expresses a variety of complement receptors, which are involved both in liver injury and repair.

3.2. Complement system and liver diseases

The role of the innate immune system in the development of liver diseases has been extensively studied (Bode et al. 2005; Hoek et al. 2006; Qin and Gao 2006). In addition to systemic effects on lipid metabolism, C3a and also the related anaphylatoxin C5a have recently been suggested to participate in the process of liver regeneration via activation of nuclear factors and cytokines (Strey et al. 2003). Both C3a and C5a mediate LPS-induced pathogenic insults by stimulating the production of prostanoids and pro-inflammatory cytokines by liver Kupffer cells (Puschel et al. 1993; Schieferdecker et al. 1997). C3 or C5 deficiency resulted in diminished liver regeneration after partial hepatectomy. This could be restored by C3a and C5a reconstitution (Strey et al. 2003). Severely defective liver regeneration was found in C5- and C3- or C3a receptor-deficient mice after CCL_4-induced liver damage, which could be restored by murine C5, C5a or C3a (Mastellos et al. 2001; Markiewski et al. 2004).

There is evidence for the role of complement system in the pathogenesis of liver fibrosis. The involvement of C5 and C5aR in liver fibrosis has been shown using A/J inbred mouse strain that carries a 2-bp deletion of the C5 gene. This strain is resistant to liver fibrosis and develops low level of fibrosis after CCL_4 treatment (Hillebrandt et al. 2005). The significance of C5 in liver fibrosis is supported by the induction of fibrosis-susceptible phenotype by the introduction of C5 deficiency into the C5 sufficient strain, and by the attenuation of liver fibrosis after blockade of the C5 receptor (C5aR1). Stimulation of C5aR1, which is expressed on the stellate cells, by C5a upregulated fibronectin expression (Schlaf et al. 2004).

In patients with HBsAg-positive hepatitis and in primary biliary cirrhosis increased C3d concentrations and changes in the serum concentrations of the complement components consistent with activation of the CP and AP have been found (Munoz et al. 1982). A decreased total hemolytic complement activity and low concentrations of C3, C4, C5, factor B, and of the regulatory proteins factors I and H were observed in the patients with liver disease due to alcohol or acetaminophen toxicity (Ellison et al. 1990). This impairment in complement function could contribute to impaired antibacterial host defense of patients with chronic hepatic disease.

Complement has been shown to be an important factor in the pathogenesis of the ischemia/reperfusion (I/R) liver injury (Chan et al. 2003). Reperfusion of the ischemic liver may induce activation of complement. Activated products of the complement components have been detected in plasma after liver I/R of pigs and humans (Straatsburg et al. 2000; Bergamaschini et al. 2001). Depletion of serum complement before ischemia in rats has been shown to attenuate superoxide generation by Kupffer cells and the accumulation of neutrophils in the liver during I/R, thereby suppressing injury (Jaeschke et al. 1993). Inhibition of the complement cascade by soluble complement receptor type 1 (sCR1) after liver I/R significantly reduced endothelial complement C3 deposition and liver injury (Chavezcartaya et al. 1995). In addition, in the liver I/R model, endothelial cell integrity in the liver was preserved in transgenic mice overexpressing the human C1 inhibitor (Inderbitzin et al. 2004).

There is accumulating evidence suggesting a role for complement system in the development of ALD. Contribution of C3 to the TG accumulation in the liver and significance of C5 in inflammation and injury have recently been demonstrated in $C3^{-/-}$ and $C5^{-/-}$ mice fed the ethanol diet (Pritchard et al. 2007).

V SUMMARY OF THE REVIEW

A large body of evidence suggests a role for the complement system in injury and in repair processes. Complement system can trigger tissue damage by inflammation or contribute to homeostasis via clearance and disposal of injured cellular material. It is suggested that complement can also contribute to the development of alcoholic liver damage. We postulated that chronic ethanol consumption can disturb homeostasis of the complement system and thus complement activation is involved in the pathogenesis of ALD. TNF-α, ROS and LPS can injure hepatocytes and convert them into potential activators of complement. Ethanol can also lead to desialylation of protective glycoproteins on cell surface and activation of the AP. Loss of terminal sialic acid from carbohydrates could also lead to exposure of mannose or N-acetyl galactose, which can activate the LP of complement. Ethanol-induced proteins or lipid modifications could activate complement either directly or via formation of antibodies that can activate the CP. Ethanol can interact with soluble and membrane bound factors, such as FH, DAF, MCP and CD59. Loss of protection could lead to complement activation and tissue damage.

Several important questions are still open. Damaging or protective functions of the complement system have been implicated in alcohol liver injury and what kind of complement-associated factors and molecular mechanisms are involved in the progression of ALD? Disturbances in the clearance and waste disposal functions of complement may be detrimental and could predispose complement-deficient individuals to alcohol-induced liver damage. Studying how C6 deficiency (in a C6-deficient rat strain, which is unable to generate the MAC) affects experimental alcohol liver injury should increase our understanding of how the complement TP components interact with protective functions against alcohol in the liver. If complement activation contributes to ethanol-induced fatty liver, then mice lacking C3 should be protected from ethanol-induced steatosis. By using a clinically relevant rodent model (C3-deficient knock-out mice) it is possible to examine the role of complement in ALD and the cellular regulatory activities and pathogenetic mechanisms that could sensitize the liver tissue to complement-mediated damage.

VI AIMS OF THE STUDY

Although ALD is recognized as a major and increasing world-wide health problem, its pathogenetic mechanisms are still poorly understood. The fact that only a minority of chronic alcohol abusers ever develop advanced ALD demonstrates that predisposing or disease promoting factors unrelated to ethanol metabolism must contribute to or participate in the development of ALD. The immune system participates in inflammatory reactions and elimination of invading foreign, apoptotic or injured cells. The present work was designed to clarify some of the immune mechanisms that are involved in the pathogenesis of ALD, with particular emphasis on the role of complement system, which is part of the both innate and acquired immune systems.

The specific aims of the present study were:

1. To analyse complement activation and complement component gene expression in livers of chronically ethanol-treated rodents.
2. To study how the absence of the terminal complement pathway (C6 deficiency) affects development of alcoholic liver injury.
3. To study how complement C3 deficiency affects alcohol-induced liver steatosis.
4. To study how ethanol and C3 deficiency affect expression of genes associated with lipid metabolism.

VII MATERIALS AND METHODS

7.1. Animals and experimental design

Normal male PVG/c rats (*C6*$^{+/+}$) and complement C6-deficient PVG/c rats (*C6*$^{-/-}$) were used in study **I**. Twelve rats from both strains received ethanol containing (5%) high-fat/low-carbohydrate liquid diet for 6 weeks (Lindros and Järveläinen 1998). Normal male C57BL/6 *C3*$^{+/+}$ and *C3*$^{-/-}$ mice (Horst, the Netherlands) were used in studies **II, III** and **IV.** All mice received a diet based on a liquid diet protocol, further modified into a gel by addition of agar (Bykov et al. 2004). Twelve mice from both strains received ethanol contained diet for 6 weeks. Pair-fed controls received control diet. In an acute ethanol exposure experiment C57BL/6 and *C3*$^{-/-}$ mice were given one dose of ethanol (5g/kg; 20% solution) for four hours intragastrically.

7.2. Laboratory methods

The table (Table 2) below lists the experimental methods used in this thesis with a reference by Roman numerals to the original publication in which they were described.

Table 2. Method used in the thesis

Method	Publication
Serum and liver adiponectin determination	**III**
Plasma and liver TNF-α and IL-10 assay	**I, III**
Fractionation of serum lipoproteins	**III**
Serum phospholipid transfer protein (PLTP) activity assay	**III**
Determination of protein concentration	**III**
Serum apolipoprotein A-I (apoA-I) measurement	**III**
Blood ethanol measurement	**I, II**
Plasma alanine aminotransferase (ALT) assay	**I, II, III**
Liver triglyceride measurement	**I, II, III**
Liver malondialdehyde determination	**I**
Examination of liver histopathology	**I, II, III**
Immunofluorescence staining and microscopy	**I, III**
Total RNA isolation	**I, III, IV**
RT-PCR analysis	**I, III, IV**
RT-PCR analysis (TaqMan protocol)	**III, IV**
Microarray gene expression assay	**III, IV**

Analytical methods

Plasma and liver adiponectin concentrations were measured by using a Quantikine ELISA kit (R&D Systems Inc., NY, USA), which recognizes both low- and high-molecular weight adiponectin. Liver adiponectin was assayed from diluted (Calibrator Diluent; 1:1000) supernatants obtained after centrifugation of liver homogenates.
Plasma TNF-α, IL-10 and PGE_2 concentrations were measured by Quantikine ELISA kits (R&D Systems Inc., NY, USA). For liver cytokine assay about 200 mg frozen liver tissue was homogenized in 25 mM Hepes buffer and centrifuged at 20,000*g* for 15 min at 4 °C. TNF-α and IL-10 levels in supernatant were measured by ELISA kits.
Serum lipoproteins were fractionated by FPLC. For each group two or three 200 µl serum samples pooled from 4 mice were analyzed. Serum phospholipid transfer protein (PLTP) activity was measured using a radiometric assay and serum apolipoprotein A-I (apoA-I) levels by sandwich ELISA.
The blood concentration of ethanol was measured by head-space gas chromatography. Plasma alanine aminotransferase (ALT) activity was assayed by commercial kit (Boehringer Mannheim, Germany). For assay of liver TG (as glycerol), 1 ml of liver methanol-chloroform mixed homogenate was washed with sodium chloride, the resultant extract was dried and dissolved in 200 µl of tetraethyl ammoniumhydroxide/95% ethanol (1:28). After incubation at 60°C for 30 min the extracts were subjected to hydrolysis by mixing with 200 µl 50 mM HCl. Glycerol was measured enzymatically by using commercial kits (Boehringer-Mannheim, Germany). The liver concentration of malondialdehyde, an indicator of lipid peroxidation, was determined by the thiobarbituric method.

Liver histopathology

Formalin-fixed liver samples embedded in paraffin were cut in 6 µm sections and stained with hematoxylin/eosin. Liver pathology was evaluated blindly. Steatosis was graded from 0 - 5 as follows: 1 = < 20% of cells containing fat, 2 = 21 - 40%, 3 = 41 - 60%, 4 = 61 - 80%, 5 = > 80%. Inflammatory cell infiltration was graded from 0 - 5 and based on the average of 10 randomly selected low-power fields. A single inflammatory cell = 1 point; an inflammatory focus consisting of 2 - 5 cells = 3 points;

and an inflammatory focus of more than 5 cells = 5 points. The total pathology score was calculated from the sum of steatosis and inflammation scores.

Immunofluorescence microscopy analysis

Liver tissue samples for indirect immunofluorescence (IFL) microscopy were quickly frozen on dry ice. Frozen sections (6 µm) were fixed with cold (- 20°C) acetone for 10 min. Sections were washed 3 times with phosphate-buffered saline (PBS, pH 7.4) and incubated for 30 min at +22°C with the primary antibodies. After three washes with PBS the sections were incubated with green fluorescein Alexa Fluor 488 goat anti – rabbit IgG antibody or Alexa Fluor 546 red fluorescein goat anti–mouse IgG antibody (Molecular Probes, Inc, Eugene, OR, USA). The slides were mounted with Immumount (Shandon Lipshaw, Pittsburgh, PA, USA). Antisera against rat C1, C8, C9 and CD59 were obtained from Prof. B.P. Morgan (University of Wales College of Medicine, Cardiff, Wales). Anti-rat C3 and anti–rat albumin antisera were purchased from Cappel Corporation (Malvern, PA, USA) and anti-Crry antibody from Pharmingen (San Diego, CA, USA). To test for non-specific reactions, control experiments without primary antibody were performed or rabbit anti–rat albumin antibody was used. The sections were analyzed with an Olympus BX-50 fluorescence microscope and photographed with a Hamamatsu Orca IIIm CCD-camera (Hamamatsu Photonics K.K., Mamatutsu City, Japan) and Openlab software (Improvision Corporation, MA, USA). In the IFL analysis control incubations were done either by omitting the primary antibody, by using nonimmune sera or by using antisera with known specificities other than those of the first antibodies.

RT-PCR analysis

Total RNA was isolated from liver samples (30 mg) using an Rneasy® Mini kit (Qiagen GmbH, Hilden, Germany). RNA quality was estimated by using the RNA 6000 Nano assay in the Agilent Bioanalyzer monitoring for ribosomal S28/S18 RNA ratio and the total concentration of RNA was determined spectrophotometrically (A_{260}). First-strand cDNA was transcribed from 1 µg RNA using Promega´s Reverse Transcription system according to the manufactureris instructions (Madison, WI, USA). PCR was performed using a PTC-200 Peltier Termal cycler (MJ Research).

The PCR products were quantified by anion exchange HPLC and expressed relative to that of β-actin mRNA.

In the experiments with microarray gene expression assay, changes in expression of selected genes were verified by real-time PCR analysis (TaqMan protocol, Applied Biosystems). First-strand cDNA was transcribed from 1 µg RNA (extracted as described above) using High-Capacity cDNA Archive Kit (Applied Biosystems) with random primers. The cDNA was used as a template for the subsequent PCR carried out in triplicate. The expression levels were analyzed using pre-designed TaqMan Gene Expression assays probes and the Applied Biosystems 7300 Real Time PCR System. Relative mRNA expression levels in each experiment were normalized against the expression level of the housekeeping 18S rRNA by the comparative Ct method and fold change values calculated according to the manufacturer's instructions.

Microarray gene expression assay

Microarray transcript profiling was carried out at the Microarray Core Facility of the Medical Faculty, the University of Helsinki, on Affymetrix MOE-430A chips that contain over 22,600 probe sets including transcripts and variants from 14,484 well annotated mouse genes. Three arrays per group were probed, and each probe represented a pool of 3-4 animals. Two µg of total RNA was treated according to the conventional Affymetrix eukaryotic RNA labeling protocol. Fifteen µg of biotin labeled cRNA was fragmented according to the Affymetrix eukaryotic sample protocol. Hybridization, staining and washing was performed using the Affymetrix Fluidics Station 450 and Hybridization Oven 640 under standard conditions. Scanned images were analyzed with Affymetrix Microarray Suite 5 software. For each probe array, a per gene normalization was applied so that signal intensities were divided by the median intensity calculated using all 12 probe arrays, effectively centering the data around unity. Normalized values were then averaged over samples in each group and ratios of these were calculated. Data processing was carried out using GeneSpring 6.1 data analysis software (Silicon Genetics, Redwood City, CA, USA). The genes were assigned to various groups according to their participation in cellular processes by using Gene Set Analysis Toolkit (*http://bioinfo.vanderbilt.edu/webgestalt*).

VIII RESULTS AND DISCUSSION

8.1. COMPLEMENT ACTIVATION AND MODULATION OF COMPLEMENT GENE EXPRESSION IN THE LIVER BY CHRONIC ETHANOL UPTAKE (I, III, IV)

8.1.1. The effect of ethanol on complement component deposition

In order to study the effects of chronic ethanol intake on the complement system we examined the deposition of complement components in liver and expression of liver genes associated with the complement system in animals with alcohol-induced liver injury. A deposition of the complement components C1, C3, C8 and C9 was observed in the livers of alcohol-treated wild type rats **(I; Figs 3** and **4)**. The same tendency was found with respect to component C3 in wild type mice, receiving prolonged ethanol treatment **(III; Fig 1).** In contrast, only minimal complement component deposition was found in livers from complement C6-deficient ethanol-fed rats. Complement regulators Crry and CD59 were expressed on hepatocyte cell membranes in both wild type and C6-deficient rats (**I; Fig 3**).

Chronic alcohol intake can be considered as a continuous inflammatory stress. Many complement components belong to type II acute phase proteins that respond to inflammatory stimuli more slowly and with a smaller increase than type I acute phase proteins, like CRP. Increased levels of the early pathway complement components could thus contribute to inflammation and tissue damage. This would be in line with an increased hepatic deposition of complement components C1, C3, C8 and C9 in rats receiving a prolonged ethanol treatment (**I**, Järveläinen et al. 2002). The decrease in deposition of the examined complement components after ethanol treatment in livers of *C6*$^{-/-}$ rats was intriguing. While it is logical that C8 and C9 are not deposited in the livers of *C6*$^{-/-}$ animals, the decrease in C1q and C3 deposition could be due to the fact that in the absence of MAC less tissue debris is produced. Cell membrane injury caused by MAC is able to expose complement-activating intracellular structures such as mitochondrial membranes and intermediate filaments.

Thus, in the *C6*$^{+/+}$ rats the normal clearance function is operating and cytoskeletal deposition of C1 and C3 could indicate that injured tissue material is recognized.

8.1.2. The effect of ethanol on complement gene expression

We analyzed how chronic ethanol intake affects the expression of hepatic genes of proteins associated with the complement system in wild type and C3-deficient (*C3*$^{-/-}$) mice by microarray transcript profiling using Affymetrix MOE-430A mouse chips. As an overall trend we observed that alcohol exposure increased mRNA expression of components of both the AP and the CP. Chronic ethanol intake increased C3 mRNA expression in wild type mice **(IV; Table 1).** The expressions of transcripts for complement factor B, C1qB, C6 and factor I were lower in the livers of *C3*$^{-/-}$ mice as compared to wild type mice, while the opposite was true for factor D (adipsin) and Masp-2 (**IV; Table 1, Figs 1-3**). Ethanol up-regulated factor B, C1qA, C2 and clusterin genes, although the effect was significant only in wild type mice **(IV; Table 1, Figs 1** and **3**). In C3-deficient mice ethanol up-regulated C1qB and vitronectin but down-regulated Masp-2 expression **(IV; Figs 1** and **3, Table 1).** It should be noted that ethanol down-regulated the expression of the soluble regulators factor H and C4bp but only in wild type mice **(IV; Fig 3).** Importantly, ethanol was found to down-regulate the mRNA level of factor D, which is the least abundant, yet the rate-limiting enzyme in AP and the terminal complement components C6, C8α and C9 **(IV; Table 1, Fig 2).** mRNA expression of the membrane-bound complement regulators MCP and CD59 was somewhat higher in C3-deficient than in wild type mice (**IV; Table 1**). Ethanol feeding slightly reduced the expression of MCP and CD59 in *C3*$^{-/-}$ mice and Crry in both genotypes.

Microarray data further suggested an increased hepatic synthesis of C3 by ethanol. However, despite an increased synthesis, the enhanced deposition of C3 is most likely due to sequestering of C3 from the circulation and activation in the liver tissue, probably as a response to cellular damage by alcohol. Injured tissue components may covalently deposit C3b after local complement activation. Thus, an increased C3 synthesis by ethanol could be indirect, induced by cytokines responding to increased LPS infiltration from the alcohol-induced “leaky” gut. This is supported by the fact that both LPS and IL-6 were found to up-regulate C3 gene expression in HepG2 cells (Wright et al. 2001). In addition, TNF-α has been found to up-regulate the expression and production of C3 in rat glomerular endothelial cells (Sheerin et al. 1997). However, in patients with alcoholic liver cirrhosis low plasma levels of the complement proteins C3 and C4 are commonly found (Calamita and Burini 1995).

This might be attributed either to the hepatic disease itself, to ethanol action and/or to malnourishment of the patient. It should be noted that even an acute single intraperitoneal injection of ethanol could induce a selective and prolonged alteration in complement-mediated immune clearance, with a decrease in complement-mediated sequestration of opsonized cells and in complement-dependent phagocytosis in mice (Messner et al. 1994). Chronic ethanol ingestion in mice is also associated with an initial decrease followed by a small rebound increase in complement-mediated clearance of injured cells (Messner et al. 1993).

Ethanol treatment increased the expression of factor B, which plays an important role in the activation of the AP. The inflammatory cytokines, in particular TNF-α and IFN-γ, are critical in regulating factor B gene expression in macrophages. LPS has been found to induce factor B promoter activity through the NF-kappa B cis-binding site (Huang et al. 2002). In contrast to factor B, ethanol markedly down-regulated the expression of factor D. This effect of ethanol on factor D could efficiently down-modulate AP activity, unless the decreased synthesis in the liver is not compensated for by an increased factor D production in the adipose tissue. The disparate effects on factor B and factor D might reflect tissue-specific responses in the liver and fat cells. It is possible that down-regulation of factor D synthesis could also reflect effects of ethanol on fat cells. The high expression of factor D in *C3*$^{-/-}$ mice fed with high-fat diet, which caused marked liver fatty infiltration, is in accordance with the data on induction of components of the AP (complement C3, properdin and factor D) in the livers of mice fed with a fat diet (Recinos et al. 2004). Although factor D is the rate-limiting enzyme of the AP it is not, unlike e.g. factor B and C3, consumed during AP activation. The changes in *C3*$^{-/-}$ mice receiving a high-fat diet were accompanied by a down-regulation of factor B. This notion is in line with the previous results on down-regulation of hepatic transcription of factor B and its CP analogue C2 in animals on high-fat diet (Recinos et al. 2004). Overall our results indicate that the activity of AP is intimately related to lipid metabolism and influenced by alcohol.

Chronic ethanol consumption also increased the expression of early CP components. It should be noted that C2 and factor B are homologous proteins (serine esterases), both encoded in the MHC class III region in chromosome 6. Ethanol regulated their expression in a similar manner. Of interest, LPS, IL-1 and TNF-α also increased C2 synthesis in human fibroblasts (Kulics et al. 1994).

Chronic ethanol intake down-regulated the expression of early pathway soluble component regulators (C4bp and factor H) in *C3*$^{+/+}$ but not in *C3*$^{-/-}$ mice and increased the expression of TP regulators (clusterin and vitronectin). The lower expression of factor I in *C3*$^{-/-}$ mice may be a consequence of the C3 deficiency, since the C3b inactivating enzyme factor I is not needed. Reduced levels of complement regulators in *C3*$^{+/+}$ mice would lead to an increased vulnerability to complement auto-attack, particularly in the liver. By promoting complement activation alcohol could thus accelerate the progression of liver steatosis and inflammation. The reduced expression of the complement cell-membrane-bound regulators Crry and CD59 by ethanol is in line with our findings of a similar tendency of mRNA expression of these regulators, especially of Crry, which is essential in rodents in preventing complement attack (Järveläinen et al. 2002).

Taken together, the results suggest that the induction of AP and CP components and suppression of TP components and soluble regulators by chronic ethanol consumption may contribute to alcohol-induced liver injury.

8.2. ROLE OF THE COMPLEMENT TERMINAL PATHWAY IN ALCOHOL-INDUCED LIVER INJURY (I)

8.2.1. Effect of complement C6 deficiency on histopathological changes and parameters of liver function in alcohol-treated rats

In order to evaluate whether an intact terminal pathway of the complement system aggravates or protects against alcohol-liver damage we compared the response to chronic ethanol feeding of rats genetically deficient in the complement component C6 (*C6*$^{-/-}$) with that of wild type C6-sufficient (*C6*$^{+/+}$) rats. C6 deficiency leads to an impaired assembly of MAC, and C6 and MAC have been shown to be involved in the development of various pathological conditions (Schmiedt et al. 1998; Nangaku et al. 2002; Tran et al. 2002). Consequently, we investigated the role of the TP in the inflammatory consequences of chronic alcohol intake. We observed that chronic ethanol feeding resulted in a marked mixed micro- and macrovesicular steatosis and infiltration of mononuclear inflammatory cells in the livers of both *C6*$^{+/+}$ and *C6*$^{-/-}$

animals (**I; Fig 1**). However, steatosis and inflammation were more prominent in *C6*$^{-/-}$ rats (**I; Figs 1** and **2**). The hepatic level of TG was also higher in *C6*$^{-/-}$ rats as compared to *C6*$^{+/+}$ rats. The control *C6*$^{-/-}$ rats seemed to be more sensitive to the high-fat diet, as evidenced from significantly higher TG concentration and liver to body weight ratio as compared to *C6*$^{+/+}$ controls. Changes in the plasma levels of liver ALT supported the histopathological findings; chronic alcohol feeding increased ALT values 12.5-fold in *C6*$^{-/-}$ and 6.5-fold in *C6*$^{+/+}$ rats (**I; Table 2**). Thus, chronic ethanol intake in normal complement sufficient rats resulted in a marked steatosis and inflammatory cell infiltration and enhanced the hepatic deposition of complement components examined. In complement C6-deficient rats no corresponding complement activation was observed, but the parameters reflecting hepatic changes were more marked. This suggests that complement activation up to the level of the terminal pathway may be important in the repair of alcohol-induced lesions or in their regeneration, thus carrying out a protective function.

A positive correlation between plasma C3 and liver enzyme elevations has been reported suggesting a role for complement activation in ALD (Bird et al. 1995). Complement activation is known to worsen the pathology of several disease states (Väkevä et al. 1993; Väkevä et al. 1995; Lindsberg et al. 1996; Väkevä et al. 1998). However, recently an important role of complement as a "clean-up" system has been suggested (Collard et al. 1999; Walport 2001 (a,b); McGeer and McGeer 2002). New evidence suggests that the complement system plays an essential role in liver regeneration. Upon partial hepatectomy C3- or C5-deficient mice exhibited high mortality, liver damage, and impaired liver regeneration (DeAngelis et al. 2006). In analogy to this, a continuous efficient regenerative repair process may be crucial in preventing the slow development of irreversible liver damage by chronic ethanol intake. Conceivably, injurious stimuli (endotoxin, oxygen free radicals, alcohol/acetaldehyde protein adducts, etc) would cause complement activation and mark injured cells for clearance by phagocytes. Complement deficiency could abrogate this function and prolong the inflammatory reaction. In the repair process MAC plays a crucial role in regulating apoptosis, cell proliferation and growth (Morgan 1989 (a,b); Väkevä et al. 1998; Soane et al. 1999; Rus et al. 2001; Zwaka et al. 2003). The number of membrane–inserted C5b-9 complexes determines whether complement activation promotes or prevents cell death. Thus, the level of complement activation could determine the pathophysiological fate of affected

tissues and recovery of function in an inflammatory milieu (Rus et al. 2001). Sublytic amounts of MAC stimulate cells by activating phospholipases, protein kinase C and the production of inflammatory mediators, such as reactive oxygen metabolites, thromboxanes and prostaglandins (Morgan 1989 (a,b); Rus et al. 2001).

Thus, while specific target cells, invading microbes or nonviable host cells often are destroyed by complement activation, the adjacent bystander cells may be saved and stimulated to respond to the local injury.

8.2.2. Effect of complement C6 deficiency on inflammatory responses in alcohol-treated rats

In order to evaluate the effect of chronic ethanol on the inflammatory responses in C6-deficiency we measured the plasma levels and liver mRNA expression of cytokines. Chronic alcohol feeding led to a significant increase in the plasma concentration of TNF-α in *C6*$^{-/-}$ but not in *C6*$^{+/+}$ rats (**I; Table 2**). In contrast, while ethanol significantly increased plasma IL-10 in *C6*$^{+/+}$ rats it had no significant effect in *C6*$^{-/-}$ rats. Consequently, in alcohol fed rats, the plasma TNF-α/IL-10 ratio was 4 times higher in *C6*$^{-/-}$ than in *C6*$^{+/+}$ rats. Ethanol increased the mRNA expression of the CD14 and IL-10 in the livers equally well in *C6*$^{+/+}$ and *C6*$^{-/-}$ rats (**I; Table 3**). After alcohol feeding the mRNA expression of TNF-α receptor-1 (TNFR1) was significantly higher in *C6*$^{-/-}$ than in *C6*$^{+/+}$ rats. The plasma ratio of TNF-α and IL-10, which reflects the balance between pro- and anti-inflammatory cytokines, was closely associated with hepatic changes and correlated significantly with plasma ALT activity. IL-10 produced mainly by Kupffer cells not only suppresses the production of pro-inflammatory cytokines, but also down-regulates cytotoxicity of T-cells and the antigen-presenting function of sinusoidal endothelial cells (Moore et al. 1993; Knolle and Gerken 2000). The absence of an ethanol-induced increase in IL-10 levels in *C6*$^{-/-}$ rats could thus be of pathogenetic importance. The lower level of the anti-inflammatory IL-10 cytokine together with the increased TNF-α response to alcohol-induced injury in *C6*$^{-/-}$ rats suggests that C6 deficiency leads to an impaired tissue clearance and a stronger inflammatory response.

The observed difference in liver fat metabolism between *C6*$^{-/-}$ and *C6*$^{+/+}$ rats prompted us also to investigate the effect of ethanol on prostanoid responses. Analysis of PGE_2 from plasma did not reveal any significant effect of ethanol, nor was there any difference between *C6*$^{-/-}$ and *C6*$^{+/+}$ rats. However, ethanol caused marked up-regulation of the mRNA expression of prostaglandin E EP2 (prostaglandin E receptor) (5.4-fold) and EP4 (8.4-fold) receptors in *C6*$^{-/-}$ animals (**I; Table 4**). In contrast, no significant up-regulation was seen in *C6*$^{+/+}$ rats. PGE_2 modulates the production of TNF-α by Kupffer cells and other macrophages by increasing the intracellular cAMP level through G-protein-coupled EP2 and EP4 receptors. The stronger up-regulation of these receptors in livers from ethanol-treated *C6*$^{-/-}$ rats suggests that complement C6 deficiency sensitizes the liver to pro-inflammatory cytokines. These effects may be associated with the more massive ethanol-induced steatosis and the higher TG concentration in control *C6*$^{-/-}$ than in *C6*$^{+/+}$ rat livers. Indeed, Kupffer cells seem to play an important role in alcohol-induced steatosis, which sensitizes the liver to further damage (Enomoto et al. 2000; Stewart et al. 2001).

Thus, in animals with a deficient TP (*C6*$^{-/-}$) parameters indicating alcohol-induced hepatic damage are more marked than in normal rats. This suggests that a malfunctioning response to injury predisposes to stronger inflammatory reactions and accelerates the development of injury. An intact terminal pathway of the complement system may have an important function in the prevention and/or repair of alcohol-induced liver lesions.

8.3. ROLE OF ALTERNATIVE COMPLEMENT PATHWAY IN ALCOHOL-INDUCED LIVER STEATOSIS (II, III)

8.3.1. Effect of complement C3 deficiency on histopathological changes, liver function and lipid parameters in alcohol-treated mice

In order to investigate the role of complement in alcohol-induced steatosis and other signs of liver damage we analysed normal mice in comparison to mice lacking the C3 complement component. The hepatic effects of both control and ethanol diet were

clearly different between wild type *C3*$^{+/+}$ and *C3*$^{-/-}$ mice. The high-fat diet by itself caused marked macrovesicular steatosis in *C3*$^{-/-}$ mice, in contrast to *C3*$^{+/+}$ mice (**II; Fig 1, Table 1**). The strain-specific diet effect was also seen as a significantly increased level of TG in the liver (**II; Fig 2**). As a consequence, the relative liver weight was also higher in *C3*$^{-/-}$ than in *C3*$^{+/+}$ mice and in animals on control diet, the serum ALT activity was significantly higher in *C3*$^{-/-}$ than in *C3*$^{+/+}$ mice (**II; Table 1**, **Fig 2**). In *C3*$^{+/+}$ mice, the ethanol diet caused significant increases in liver steatosis, TG concentration and the liver/body weight ratio. Serum ALT activity and the concentration of MDA, which reflects lipid peroxidation, were doubled. In *C3*$^{-/-}$ mice, which received ethanol the steatosis score was even lower than in mice on ethanol-free diet, and there was no effect of ethanol diet on liver TG.

To elucidate whether the aberrant effect of ethanol on liver lipids in C3$^{-/-}$ mice was specific to the high-fat diet composition combined with chronic ethanol exposure, a separate study was undertaken. *C3*$^{+/+}$ and *C3*$^{-/-}$ mice kept on chow diet were intragastrically intubated and given a single intoxicating dose of ethanol. Four hours later the concentration of TG in the liver had increased by 138% in the normal *C3*$^{+/+}$ mice as compared to mice given water. In contrast, in livers of *C3*$^{-/-}$ mice the increase was only 64% (**II; Fig 3**). Thus, following both acute and chronic ethanol intake *C3*$^{+/+}$ and *C3*$^{-/-}$ mice respond differently, implying a role for C3 and the complement system in regulating liver steatosis.

Ethanol-induced fatty infiltration sensitizes the liver to subsequent inflammatory and necrotic changes (Lieber 2004). The inflammatory response includes activation of the complement system and generation of inflammation-inducing anaphylatoxins (Nielsen et al. 2000; Oksjoki et al. 2003; Ogden and Elkon 2006). The complement component C3 has been shown to affect lipid homeostasis via its cleavage product $C3a_{desArg}$ (acylation-stimulating protein, ASP) (Cianflone et al. 1999; Cianflone et al. 2003). C3-deficient animals that cannot generate ASP exhibit a compromised regulation of adipocyte lipid storage and TG synthesis (Baldo et al. 1993; Murray et al. 1999). Consequently, the complement system may also be involved in the development of alcohol-induced steatosis. Our data that in C3 deficient mice ethanol exposure does not increase the accumulation of TG, a phenomenon normally seen in rodents as well as in man suggests that C3 contributes to ethanol-induced liver fatty infiltration (Stewart et al. 2001; Persson et al. 2004). Our data were supported by the

recent observation that the *C3*$^{-/-}$ mice were protected against ethanol-mediated steatosis (Pritchard et al. 2007). Thus, an intact complement system, and especially the presence of C3, contributes to liver damage rather than being protective (**II**). This contribution to damage seems to be indirectly mediated via steatosis, which is considered to sensitize the liver to inflammatory attack. This concept is supported by our findings that in *C3*$^{+/+}$ mice, but not in *C3*$^{-/-}$ mice, chronic ethanol administration increased serum ALT activity and liver MDA concentration. MDA is an indicator of the degree of lipid peroxidation, which gives rise to reactive oxygen radicals injurious to cells. The fact that C3 has a different and more central role in the complement cascade than C6 may explain why C6 deficiency seemed to aggravate rather than protect against damage.

Thus our results suggest a dual role for the complement system where, on one hand, it contributes to inflammation and tissue damage and, on the other hand, participates in the clean-up process and tissue repair and/or regeneration.

Many studies indicate that the AP of the complement system influences lipid metabolism. Thus, in patients with an antibody against the AP C3 convertase C3bBb, called C3 nephritic factor (C3Nef), partial lipodystrophy occasionally accompanies membranoproliferative glomerulonephritis type II (Sissons et al. 1976). C3Nef causes hypercatabolism of the AP and drastically lowers C3 levels (Misra et al. 2004). Hypercatabolism of C3 and consequent depletion of C3 thus seem to be related to redistribution of fat from peripheral tissue to the liver and the lower abdominal area.

Complement C3 activation leads to the formation of C3a, which is subsequently converted to C3a/desarg, alias ASP, following removal of the C-terminal arginine. ASP has been suggested to participate in the clearance of postprandial TG and to promote uptake of free fatty acids to peripheral fat tissue. It is therefore possible that the absence of C3 and ASP reduces the risk for peripheral obesity but allows steatosis in the liver, as we have observed in our present mouse model. In the presence of ethanol, however, the situation seems to be somewhat different. In livers of the *C3*$^{+/+}$ mice, but not in the *C3*$^{-/-}$ mice, ethanol increases fat accumulation and this is accompanied by more hepatotoxic and inflammatory changes. This suggests that an intact complement system contributes to these changes. Such changes could be a consequence of enhanced production of inflammatory signals by Kupffer cells

activated by circulating LPS in response to ethanol. Although moderate activation of Kupffer cells can contribute to repairing injury, excessive activation may be detrimental and lead to aggravation of injury.

Ethanol treatment increased steatosis and TG selectively in wild type mice (**III; Table 1**). For several of the lipid and lipoprotein parameters analyzed, ethanol treatment caused similar changes in *C3*$^{+/+}$ and *C3*$^{-/-}$ mice (**III; Table 1**). While ethanol had no significant effect on serum TG, serum total cholesterol levels and the activity of PLTP were significantly reduced in both genotypes. Analysis of the lipoprotein profiles suggested that ethanol reduced apoA-I levels in the eluted HDL fractions slightly more in *C3*$^{+/+}$ (18%) than in *C3*$^{-/-}$ mice (6%). The different liver lipid response in *C3*$^{+/+}$ and *C3*$^{-/-}$ mice prompted us to measure adiponectin from serum and liver. Liver adiponectin levels in animals on control high-fat diet were significantly lower in *C3*$^{-/-}$ than in *C3*$^{+/+}$ mice (**III; Fig 2**). In *C3*$^{+/+}$ mice ethanol reduced serum adiponectin levels, an effect that was more apparent after acute ethanol challenge (**III; Fig 2**). In contrast, in *C3*$^{-/-}$ mice chronic ethanol challenge increased the level of adiponectin both in serum and in the liver tissue.

The significant increase by ethanol of serum and liver adiponectin concentration in C3$^{-/-}$ mice may thus contribute to the observed reduction in hepatic steatosis. The simultaneous down-regulation of adiponectin receptor 2 mRNA expression may represent a feed-back regulatory response to adiponectin (**III**, **Fig 4**). In C3 deficiency the ethanol-induced increase in adiponectin is accompanied by that of the homologous complement protein C1q B-chain, in a possible concerted response-to-injury action. This response could have a protective function or a role in the clearance of lipid particles or injured cells. Adiponectin affects energy homeostasis and insulin sensitivity via activation of AMP kinase and PPAR-α, thus increasing lipid β-oxidation and decreasing tissue TG levels (Yamauchi et al. 2003). In Zucker rats high levels of adiponectin were associated with alleviated nonalcoholic fatty liver and reduced expression of TNF-α (Nagao et al. 2005). Adiponectin also has anti-inflammatory actions and its therapeutic delivery reduces fatty liver disease in rodents (Xu et al. 2003; Thakur et al. 2006).

8.3.2. Effect of complement C3 deficiency on the expression of liver genes associated with lipid metabolism in alcohol-treated mice

To better understand how complement C3 affects lipid metabolism during chronic alcohol exposure we analyzed expression of genes associated with lipid and lipoprotein metabolism by microarray transcript profiling. At the gene expression level, the effect of ethanol on apolipoproteins was similar in *C3*$^{+/+}$ and *C3*$^{-/-}$ mice (**III; Fig 3**). The effects of ethanol treatment on the expression of genes of fatty acid metabolizing enzymes were in general similar in both genotypes (**III; Table 2**). Chronic ethanol intake increased the expression of acyl-CoA dehydrogenase, but down-regulated acetyl-carboxylase-beta, carnitine palmitoyltransferase I, acyl-CoA synthetase and hydroxyacyl-CoA dehydrogenase type II transcripts as well as two fatty acid binding proteins (**III; Table 2**). The hepatic expression of several other genes exhibited patterns reminiscent of the effect on fatty infiltration in *C3*$^{-/-}$ mice. Thus the expressions of both AdR2 and ADRP were significantly down-regulated by ethanol exclusively in *C3*$^{-/-}$ animals (**III; Fig 4**). The expression of SREBP-1 was significantly higher in *C3*$^{-/-}$ mice while ethanol significantly reduced PPAR-α mRNA exclusively in *C3*$^{+/+}$ mice. As was observed at the protein level, reduction by ethanol feeding of mRNA for PLTP was similar in both genotypes (**III; Fig 4**). The expression of PLD1 was significantly reduced in *C3*$^{-/-}$ mice but this effect was counteracted by ethanol treatment. The expression of ADRP, a lipid droplet protein, was strongly induced in cells with increased lipid load. ADRP-deficient mice have reduced hepatic TG and resist diet-induced fatty liver, suggesting that ADRP is involved in TG deposition (Chang et al. 2006). Our finding that ADRP was significantly down-regulated by ethanol only in C3$^{-/-}$ mice, with reduced steatosis, is consistent with this notion. The increased expression of SREBP-1 in *C3*$^{-/-}$ mice could be related to the steatotic effect of the diet in these mice. The SREBP-1c variant predominating in the liver is lipogenic and could act via up-regulation of FAS expression (Horton et al. 2002; Eberlé et al. 2004). However, SREBP-1c does not seem to be critically involved in ethanol-induced steatosis here, since in contrast to an earlier report (You and Crabb 2004 (a)), ethanol had no significant effect on its expression in either strain.

Our observation that ethanol down-regulated the mRNA expression of PPAR-α in *C3*$^{+/+}$ but not in *C3*$^{-/-}$ mice may be of particular relevance. PPAR-α activation increases liver disposal of fatty acids and PPAR-α knock-out animals develop fatty liver and obesity (Costet et al. 1998; Kersten et al. 1999; Jump et al. 2005). Furthermore, ethanol feeding has been shown to compromise induction of PPAR-α-regulated enzymes, thus contributing to the development of fatty liver (Fischer et al. 2003). These data are in line with the present study, suggesting an important role of PPAR-α expression in the development of alcohol-induced liver steatosis.
PLD1 is important in protein kinase C signaling and in the assembly of VLDL (Asp et al. 2000). This suggests the possibility that up-regulation of the PLD1 gene by ethanol in the *C3*$^{-/-}$ mice could enhance hepatic VLDL secretion, thus affecting the level of steatosis in these mice.

The results indicate that the complement system is involved in the regulation of diet- and alcohol-induced fatty liver. Our observations in *C3*$^{-/-}$ animals support the notion that adiponectin, SREBP-1 and ADRP are important in lipid homeostasis, regulating fatty acid synthesis and uptake in the liver. Enhanced adiponectin levels, increased phospholipase D1 expression and reduced ADRP expression appear to protect against hepatic lipid deposition. Adiponectin may also, together with the complement component C1q, be involved in processing of lipid particles or materials released from injured cells. Ethanol intake leads to cell injury and complement C3-dependent steatosis with both direct and compensatory changes in several genes involved in lipid metabolism and clearance functions.

IX CONCLUDING REMARKS

The studies described in this thesis demonstrate the involvement of the complement system in the development of inflammation and steatosis in alcoholic liver disease.

To define the complement pathways involved, we investigated the expression of complement component and regulator gene transcripts by microarray analysis in the livers of alcohol-treated mice. Ethanol down-regulated mRNA levels of adipsin and of the TP complement components C6, C8α and C9. The up-regulation of complement factor B, C1qA, C2, C3 and clusterin and down-regulation of factor H and C4bp by ethanol was significant in wild type animals. These findings suggest that an induction of CP and AP and suppression of TP and complement regulator expression by chronic ethanol may be additional mechanisms of alcohol-induced liver injury. This would suggest an increased tendency for opsonophagocytosis and inflammation, and their possible involvement in the development of ALD.

We also assessed the role of the TP in alcohol-induced liver damage in complement C6-deficient rats. Our results indicate that a deficient TP predisposes to tissue injury and promotes a pro-inflammatory cytokine response. The hepatic histopathological changes, the liver weight increase and the elevation of the plasma pro-/anti-inflammatory cytokine ratio were more marked in $C6^{-/-}$ than in wild-type rats.

Liver steatosis, a typical early consequence of alcohol exposure, sensitizes the liver to more severe inflammatory and fibrotic changes. On the other hand, activation of the key complement component C3, a central player in causing inflammation and tissue damage is also known to be involved in the regulation of lipid metabolism. This prompted us to study the development of alcoholic liver steatosis in mice lacking C3. Ethanol-containing diet caused marked macrovesicular steatosis and increased the liver TG level in $C3^{+/+}$ mice. In contrast, ethanol diet tended to reduce steatosis and had no further effect on liver TG in $C3^{-/-}$ mice. This suggests that the complement system and particularly its component C3 contribute to the development of alcohol-induced fatty liver.

To understand the underlying molecular mechanisms of the involvement of C3 in ethanol-induced steatosis we analyzed lipid parameters and liver gene expression

profiles in *C3*$^{+/+}$ and *C3*$^{-/-}$ mice fed with an alcohol-liquid diet. Ethanol treatment increased serum and liver adiponectin levels but down-regulated transcripts of lipogenic enzymes, adiponectin receptor 2 and adipose differentiation-related protein and up-regulated phospholipase D1 exclusively in *C3*$^{-/-}$ mice. This suggests an important cross-talk between the complement system and lipid regulators in ethanol-induced steatosis. We propose that these ethanol-induced alterations observed in C3-deficient mice contribute to protection against fatty infiltration and subsequent inflammatory processes in the livers of these mice.

In summary, deposition of complement components C1, C3, C8 and C9 was observed in the livers of animals with alcohol-induced liver injury, and a deficient complement TP was found to predispose to tissue injury and promote a pro-inflammatory cytokine response, whereas C3 deficiency contributed to protection against fatty infiltration. These findings may have implications for the therapy of alcohol-induced liver injury and its consequences.

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