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# Non-alcoholic fatty liver disease (NAFLD) potentiates autoimmune hepatitis in the CYP2D6 mouse model

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) and its more severe development non-alcoholic steatohepatitis (NASH) are increasing worldwide. In particular NASH, which is characterized by an active hepatic inflammation, has often severe consequences including progressive fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). Here we investigated how metabolic liver injury is influencing the pathogenesis of autoimmune hepatitis (AIH). We used the CYP2D6 mouse model in which wild type C57BL/6 mice are infected with an Adenovirus expressing the major liver autoantigen cytochrome P450 2D6 (CYP2D6). Such mice display several features of human AIH, including interface hepatitis, formation of LKM-1 antibodies and CYP2D6-specific T cells, as well as hepatic fibrosis. NAFLD was induced with a high-fat diet (HFD). We found that pre-existing NAFLD potentiates the severity of AIH. Mice fed for 12 weeks with a HFD displayed increased cellular infiltration of the liver, enhanced hepatic fibrosis and elevated numbers of liver autoantigen-specific T cells. Our data suggest that a pre-existing metabolic liver injury constitutes an additional risk for the severity of an autoimmune condition of the liver, such as AIH.



## 1. Introduction

The prevalence of NAFLD is estimated to range somewhere between 20 and 30% in the western world [1,2]. In obese individuals and in subjects with type 2 diabetes (T2D) the prevalence is much higher, namely 80–90% and 30–50%, respectively [1]. In parallel to obesity and T2D the prevalence and incidence of NAFLD is increasing worldwide. Besides the further development to NASH, NAFLD has been associated with an increased risk to develop cirrhosis and HCC [2,3]. However, there is not much knowledge about the consequences of NAFLD on the development of autoimmune liver diseases, such as autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC). Obesity has been reported as a risk factor for the development of many autoimmune diseases including rheumatoid arthritis (RA), multiple sclerosis (MS) and psoriasis [4]. Furthermore, obesity worsens the course of RA, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD) and psoriasis and also impairs the treatment of RA, SLE, IBD and psoriasis [4]. Thus, it seems reasonable to assume that an organ that already displays the consequences of obesity, such as NAFLD in the liver, is prone to chronic inflammation and autoimmunity and/or may potentiate an autoimmune condition. NASH can be considered as the inflammatory implication of NAFLD with cellular infiltrations by immune cells and the intrahepatic generation of inflammatory factors [2,5]. A possible autoimmune feature of NASH has been suggested on basis of data obtained from a series of patients with NASH and from three different animal models of NASH [6]. A large fraction of NASH patients (26 out of 54) also manifested signs of AIH or PBC, such as

*Abbreviations:* NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; AIH, autoimmune hepatitis; CYP2D6, cytochrome P450 2D6; LKM-1, type 1 liver/kidney microsomal antibodies; HFD, high-fat diet; T2D, type 2 diabetes; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; RA, rheumatoid arthritis; MS, multiple sclerosis; SLE, systemic lupus erythematosus; IBD, inflammatory bowel disease; ANA, anti-nuclear antibodies; AMA, anti-mitochondrial antibodies; SMA, anti-smooth muscle autoantibodies; SC, standard chow; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HSC, hepatic stellate cell; MCD, methionine and choline deficient.

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interface hepatitis and generation of anti-nuclear (ANA) or anti-mitochondrial antibodies (AMA) [6]. In addition, up to one third of female mice with induced NASH also displayed the generation of ANA or portal inflammation with bile duct damage [6]. Despite these data not much is known about a possible influence of NAFLD or NASH on the susceptibility for AIH or the severity of the disease. In most cases such preexisting conditions had an impact on AIH diagnosis and were included into the wide spectrum of overlap syndromes [7].

Here, we wanted to investigate whether NAFLD may influence the severity of experimental AIH as a representative of autoimmune liver diseases in more detail. AIH is a severe autoimmune-mediated disease that results in the progressive destruction of the liver parenchyma and chronic fibrosis [8–12]. The clinical spectrum of AIH is ranging from an asymptomatic presentation to the display of severe symptoms indistinguishable from those of an acute viral hepatitis or with fulminant hepatic failure [8–12]. Type 1 AIH (AIH-1) is typically characterized by the presence of ANA and/or anti-smooth muscle (SMA) autoantibodies, whereas the hallmark of type 2 AIH (AIH-2) are type 1 liver/kidney microsomal autoantibodies (LKM-1) [13]. The major autoantigen recognized by LKM-1 antibodies was identified in the late 1980s as the 2D6 isoform of the large cytochrome P450 enzyme family (CYP2D6) [14,15].

For our studies we used the inducible CYP2D6 mouse model for AIH and focused on the severity of AIH and the magnitude of the liver-autoantigen-specific immune response in mice with NAFLD. In the CYP2D6 mouse model AIH-like disease is induced in wild type mice with an Adenovirus expressing the major human AIH autoantigen CYP2D6 (Ad-2D6) [16,17]. The features of AIH in the CYP2D6 model reflect many immunopathologic features of the human disease [10,12,18], including interface hepatitis, liver fibrosis and generation of CYP2D6-specific antibodies and T-cells [16,17]. We found that mice fed with a high fat diet (HFD) starting at week 4 of age and infected with Ad-2D6 after 12 weeks of HFD display an exaggerated CYP2D6-specific immune response resulting in a more severe form of AIH compared to mice that have been fed with a standard diet. We conclude from our studies that NAFLD might be a factor that is promoting and exacerbating autoimmune liver disease.

## 2. Materials & methods

### 2.1. Mice and virus

Six weeks old male C57Bl/6J mice were fed with a diet containing 10% (standard chow, SC) or 60% (high fat diet, HFD) of the caloric contents from fat during the whole experiment. Both standard chow (ssniff R/MH) and high-fat diet chow (ssniff EF acc. D12492 (I) mod.) were obtained from sniff Spezialitäten GmbH (Soest, Germany). Mice were injected with $2 \times 10^8$ pfu of Ad-2D6 (i.p and i.v) [17] after twelve weeks of diet. Ad-2D6 was generated as described elsewhere [17] and titers were determined with the Adeno-X rapid titer kit (Clontech, Palo Alto, USA). Body weight was controlled at monthly intervals. All animal experiments have been approved by the local Ethics Animal Review Broad Darmstadt, Germany (V54-19c20/15-FU-1015).

### 2.2. Blood glucose levels

Blood samples were obtained from the tail vein. Blood glucose was monitored with a dynaValeo glucometer from dynamiCARE at monthly intervals. Animals with BG levels >300 mg/dl were considered diabetic.

### 2.3. Glucose tolerance test

Mice were fasted overnight (12–16 h) and obtained a single intraperitoneal injection of glucose in sterile PBS (1.5 mg/g body weight) in the morning. Blood glucose was measured before injection and 10, 20, 30, 40, 60, 120 and 240 min post-injection.

### 2.4. Serum ALT, AST and triglycerides measurements

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and serum triglycerides were measured with the Reflotron Plus blood analysis system (Roche Diagnostics, Mannheim, Germany). Blood samples were collected with heparin coated capillaries and serum was either stored at −80 °C (AST/ALT) or immediately used (triglycerides).

### 2.5. Anti-CYP2D6 antibody determination (ELISA)

96-well microtiter plates were coated overnight at 4 °C with 0.25 mg/ml recombinant hCYP2D6 (Invitrogen, Life Technologies, Darmstadt, Germany) in 100 nM carbonate-buffer (pH 9.6) and plates were blocked with 2% FCS in PBS for 90 min at room temperature. Sera were added in PBS containing 2% FCS and were incubated for 90 min at 37 °C. The dilution series started at 1:100, followed by 1:3 dilution steps down to a dilution of 1/218,700. Alkaline-phosphatase-labelled goat anti-mouse antibody (1:2000, Southern Biotech, Birmingham, USA) was added for 90 min and the reaction was developed by addition of ECF substrate (GE Healthcare Bio-Sciences). Fluorescence intensity was determined using a Pharos FX molecular imager (Bio-Rad).

### 2.6. Hematoxylin and Eosin staining

Paraffin embedded liver sections were deparaffinzied and rehydrated before incubating the tissue in a hematoxylin bath for 8 min. The sections were washed in warm tap water for 10 min, rinsed in deionized $H_2O$ and 95% EtOH and counterstained in Eosin G/Y solution for 60 s. The sections were then dehydrated in 95% EtOH and EtOH (p.a.), cleared in xylene and mounted.

### 2.7. Sirius Red staining

Paraffin embedded liver sections were deparaffinzied and rehydrated before incubating the tissue with 100 μl Sirius Red solution (ElectronMicroscopyScience) for 1 h at RT. Afterwards, slides were washed with 0.01 N HCl and $H_2O$, dehydrated EtOH (p.a.), cleared in xylene and mounted.

### 2.8. Immunohistochemistry

Tissues were immersed in Tissue-Tek OCT (Bayer), and quick-frozen on dry ice. Using cryomicrotome and sialin-coated Superfrost Plus slides (Fisher Scientific), 7 μm tissue sections were cut. Sections were then fixed with ethanol or 50/50 ethanol/acetone at −20 °C, and, after washing in PBS, an avidin/biotin-blocking step was included (Vector Laboratories). Primary and biotinylated secondary antibodies (Vector Laboratories) were reacted with the sections for 120 min and 60 min, and color reaction was obtained by sequential incubation with avidin-peroxidase conjugate (Vector Laboratories) and diaminobenzidine-hydrogen peroxide. Primary antibodies used were: rat anti-mouse CD4, rat anti-mouse CD8 (both from BD Biosciences), rat anti-mouse CD19 (Abcam), rat anti-mouse CD11b (eBioscience), biotinylated anti-mouse CD11c (BioLegend), rabbit anti-mouse Collagen I (Millipore), rat anti-mouse F4/80 (Serotec), goat anti-mouse NKp46 (R&D Systems), mouse