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ARGINASE 2 DEFICIENCY RESULTS IN SPONTANEOUS STEATOHEPATITIS: A NOVEL LINK BETWEEN INNATE IMMUNE ACTIVATION AND HEPATIC DE NOVO LIPOGENESIS

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Abstract

BACKGROUND & AIMS—Innate immune activation has been postulated as a central mechanism for disease progression from hepatic steatosis to steatohepatitis in obesity-related fatty liver disease. Arginase 2 competes with inducible nitric oxide synthase (iNOS) for its substrate and the balance between these two enzymes plays a crucial role in regulating immune responses and macrophage activation. Our aim was to test the hypothesis that arginase 2 deficiency in mice favors progression from isolated hepatic steatosis, induced by high fat feeding to steatohepatitis.

METHODS—Arginase 2-knockout (*Arg2^{-/-}*) mice were studied for changes in liver histology and metabolic phenotype at baseline and after a short term course (7 week) feeding with a high fat (HFAT) diet. In additional experiments, *Arg2^{-/-}* mice received tail vein injections of liposome-

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encapsulated clodronate (CLOD) over a three-week period to selectively deplete liver macrophages.

RESULTS—Unexpectedly, Arg2^{-/-} mice showed profound changes in their livers at baseline characterized by significant steatosis as demonstrated with histological and biochemical analysis. These changes were independent of systemic metabolic parameters and associated with marked increase mRNA levels of genes involved in hepatic de novo lipogenesis. Liver injury and inflammation were present with elevated serum ALT, marked infiltration of F4/80 positive cells, and increased mRNA levels of inflammatory genes. HFAT feeding exacerbated these changes. Macrophage depletion after CLOD injection significantly attenuated lipid deposition and normalized lipogenic mRNA profile of livers from Arg2^{-/-} mice.

CONCLUSIONS—This study identifies arginase 2 as novel link between innate immune responses, hepatic lipid deposition, and liver injury.

Keywords

nonalcoholic fatty liver disease; steatohepatitis; arginase 2; macrophage activation; innate immunity; inflammation; liver fibrosis

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently the most common form of chronic liver disease affecting both adults and children, and is strongly associated with obesity and insulin resistance [1, 2]. One in three adults and one in ten children or adolescents in the United States have hepatic steatosis, a stage within the spectrum of NAFLD, that is characterized by triglyceride accumulation in liver cells and follows a benign non-progressive clinical course [3, 4]. Nonalcoholic steatohepatitis (NASH) is defined as lipid accumulation with evidence of cellular damage, inflammation and varying degrees of scarring or fibrosis [5]. NASH is a serious condition, as approximately 25% of these patients progress to cirrhosis and its known complications that include portal hypertension, liver failure and hepatocellular carcinoma [6–8].

NAFLD pathogenesis, and specifically disease progression to NASH, has been the subject of intense investigation over the past decade, as both diagnostic and prognostic markers and treatment options for patients with this disease remain limited [9]. A multi-hit model of NAFLD pathogenesis has emerged in recent years in which intrahepatic lipid accumulation in the setting of insulin resistance is postulated to be the first hit, which sets the stage for multiple “second hits” resulting in inflammation, hepatocellular damage and eventually fibrosis [10]. Growing evidence supports the concept that innate immune activation and increased oxidative stress in the liver are key “second hits” involved in disease progression to NASH. Activation of liver resident macrophages or Kupffer cells, at least in part by an increase in exposure to gut-derived bacterial wall components such as endotoxin, result in an increased production of pro-inflammatory cytokines as well as inducible nitric oxide synthase (iNOS) and has been proposed to play a pivotal role in this process [11, 12]. Expression of iNOS in cell types such as macrophages and hepatocytes is induced by proinflammatory cytokines [13], and iNOS has been implicated in the pathophysiology of

NAFLD [14–16]. Mammals express two isoforms of arginase, an enzyme that competes with iNOS for its substrate, arginine, designated as types 1 and 2 [17–19]. Arginase 1 is mainly expressed in hepatocytes, and mice with a disruption of arginase 1 gene die soon after birth. Arginase 2 is poorly expressed in hepatocytes, and most highly expressed in kidney, prostate, and immune cells such as monocyte/ macrophages [17, 19]. An imbalance in iNOS/arginase ratio in favor of the former may play a crucial role in regulating immune responses and macrophage activation towards a classically “M1” activated, pro-inflammatory state [20–23]. The aim of our study was to test the hypothesis that arginase 2 deficiency in mice would result in progression from isolated hepatic steatosis typically seen in diet induced obesity (DIO) to steatohepatitis. Our data identify a novel link between Kupffer cell activation and hepatic steatosis and suggest that in the presence of arginase 2 deficiency, altered immune responses precedes, and is responsible of lipid deposition in hepatocytes mainly by activation of de novo lipogenesis pathways in the liver. These findings have important implications for the pathogenesis of NAFLD and potential development of novel treatment strategies for patients with this condition.

METHODS

Animal studies

The experimental protocol has been approved by the Institutional Animal Care and Use Committee at Cleveland Clinic. Male C57BL/6 wild-type (WT) were purchased from Jackson Laboratory. The generation of *Arg2*^{-/-} mice has been described previously [24]. *Arg2*^{-/-} mice are viable and indistinguishable from WT mice. Mice were fed either a diet consisting of 5% fat (TD 2918, Harlan Laboratories, Madison, WI) or a high fat (HFAT), western type diet (consisting of 42% of Kcal from fat, TD88137, Harlan Laboratories, Madison, WI). Total body weight was recorded on a weekly basis. At indicated time points, plasma and liver tissue were collected and weighed after an overnight fast as described previously [25].

Analyses of plasma and liver metabolic mediators

Blood was collected from *Arg2*^{-/-} and WT mice after an overnight fast by cardiac puncture. Blood was spun at 2000 RPM for 15 min at 4°C, plasma drawn from the top layer, argon overlaid and stored at -80°C. Plasma assays of insulin and glucose were performed using commercially available mouse insulin ELISA (ALPCO Diagnostics, Salem, NH) and glucose assay (Cayman Chemical, Ann Arbor, MI) kits. Plasma and liver triglyceride and free fatty acid levels were measured with triglyceride-GPO liquid reagent (Pointe Scientific, Inc, Canton, MI), and free fatty acid quantification (BioVision, Mountain View, CA) kits according to manufacturers' instructions. Serum alanine aminotransferase (ALT) concentrations were measured and expressed as international units per liter (Clinical Laboratory Services, Cleveland Clinic Foundation, Cleveland, OH).

Histopathology and immunostaining

Mouse tissue was diced into 5 × 5-mm sections, immersion-fixed in PBS containing 4% paraformaldehyde for 24 h at 4°C, and embedded in paraffin. Four micrometer sections were mounted on glass slides. Hematoxylin and eosin (H&E) stained liver specimens were

evaluated by light microscopy for histopathological scoring by a hepatopathologist (BGP). Steatosis, inflammation, and ballooning were scored based on NAFLD activity score [26]. Presence of macrophage infiltration was assessed by immunohistochemical staining for F4/80. Paraffin-embedded liver sections were deparaffinized and antigen retrieval using 10 mM sodium citrate buffer was performed. Sections were incubated with primary antibody overnight at 4°C (1:50 dilution, AbD Serotec, Oxford). Subsequently, sections were incubated with a biotinylated anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA) and a Vectastain ABC Elite Kit according to manufacturer's instructions (Vector Labs). Sections were developed with ImmPACT DAB peroxidase substrate (Vector Labs) and counterstained with hematoxylin.

Oil Red O staining

Assessment of hepatic steatosis was performed by staining with Oil Red O (ORO), a fat-soluble diazo dye. Frozen liver sections (10 µm thick) were mounted on glass slides. ORO stock solution was prepared by mixing 300 mg ORO (Sigma-Aldrich, St. Louis, MO) and 100 ml 2-propanol, 99% (Fisher Scientific, Pittsburgh, PA). A working solution of 1.5:1 ORO stock solution:distilled water was then prepared. Liver slides were stained with ORO working solution for 12 minutes, after which they were washed in distilled water twice for 20 seconds and rinsed in running tap water for 10 minutes. Finally, slides were stained with hematoxylin for 45 seconds and then washed in distilled water.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from liver using RNeasy Mini kit (Qiagen, Valencia, CA). One µg RNA was reverse transcribed using random decamers and MMLV-reverse transcriptase (Applied Biosystems). Genes assayed for mRNA expression levels included inflammatory cytokines, markers of hepatic stellate cell activation, hepatic insulin signaling, and mediators of lipid trafficking and metabolism (see supplementary table 1 for primer sequences). The fold change over control samples was calculated using $2^{-\Delta\Delta CT}$ using MaxPro software (Agilent, Santa Clara, CA). 18S ribosomal RNA was used as an endogenous control.

Immunoblot analysis

Immunoblot analysis were performed as previously described.[27] Anti-Arginase 2 (ARG2, Santa Cruz, Santa Cruz, CA, USA), anti-fatty acid synthase (FAS, GeneTex, Irvine, CA, USA), and anti-stearoyl-CoA desaturase 1 (SCD-1, Cell Signaling Technology, Boston, MA, USA) antibodies were used in combination with appropriate peroxidase-conjugated secondary antibodies. Protein load was verified with an α -tubulin antibody (dilution 1:10,000) (Hybridomabank, University of Iowa) (kindly provided by M. Kaulich). Bands were visualized with the enhanced chemiluminescence reagent and digitized using a CCD camera (ChemiDoc®, Biorad, Hercules, CA, USA). Expression intensity was quantified by ImageLab (Biorad).

Liver-specific macrophage depletion

Macrophage depletion studies were done in *Arg2*^{-/-} and wild type mice using liposome-encapsulated clodronate injection as is a validated method for in vivo depletion of tissue-specific macrophages [28, 29]. Mice received four tail vein injections of 100 μ L/10 g body weight of 1 mg/ml PBS or clodronate (CLOD)-containing liposomes over a three-week period. Mice were sacrificed 24 hours after the fourth injection, at which time plasma, and liver tissue was collected.

Statistical analysis

All data were expressed as the mean $\pm$ S.D. unless otherwise indicated. Differences between groups were compared by an ANOVA analysis followed by a post hoc Bonferroni test to correct for multiple comparisons. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Arg2^{-/-} mice develop spontaneous liver injury and hepatic steatosis

Arg2^{-/-} and WT mice showed similar trends in weight gain for up to 14 weeks of age (Fig. 1A) and no evidence of systemic metabolic derangements with plasma assays of fasting triglycerides, free fatty acids, glucose and insulin (Fig. 1B). However, serum levels of alanine aminotransferase (ALT) were elevated in *Arg2*^{-/-} mice compared to WT animals (110 vs. 35 U/L, $p < 0.05$; Fig. 1D) suggesting the presence of liver injury. Indeed, H&E and ORO stained liver sections revealed notable inflammation and lipid deposition in *Arg2*^{-/-} mice compared to WT (Fig. 1C). This was associated with increased hepatic triglyceride levels (*Arg2*^{-/-} 4.9 vs. WT 2.6 mg TG/g liver, $p < 0.05$; Fig. 1E).

De novo lipogenesis (DNL) is enhanced in *Arg2*^{-/-} mice

To further explore this phenotype of hepatic steatosis in *Arg2*^{-/-} mice, the contributions of distinct processes involved in lipid trafficking and metabolism were characterized by qRT-PCR. Fatty acid uptake into and transport within the liver was assayed by CD36 or fatty acid translocase, fatty acid transport proteins (FATP) and fatty acid binding proteins (FABPs). Hepatic FABP1 mRNA expression was increased twofold, while FABP4, FATP2 and FATP5 were modestly decreased in *Arg2*^{-/-} mice compared to WT animals (Fig. 2A). Fatty acid synthesis and de novo lipogenesis (DNL) were assessed by measuring mRNA levels of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD-1), the transcription factor sterol regulatory element binding protein 1c (SREBP-1c), liver X receptor alpha (LXR- α), ATP citrate lyase (ACLY), and elongation of long chain fatty acids 2, 5 and 6 (Elovl2, 5, 6). We were able to show that hepatic mRNA expression of several enzymes involved in DNL were significantly increased in *Arg2*^{-/-} mice, with SREBP1c and SCD-1 expression increased three- and two-fold over WT controls, respectively (Fig. 2B). A significant increase was also documented for mRNA levels of LXR- α , ACLY, and Elovl2. Fatty acid oxidation, assessed by measuring mRNA levels of carnitine palmitoyl-transferase 1 (CPT-1), and acyl-coenzyme A dehydrogenase, C-4 to C-12 straight chain (ACADM), did not appear to contribute to hepatic lipid accumulation

within $Arg2^{-/-}$ mice (Fig. 2C). Markers for triglyceride export; microsomal triglyceride transfer protein (MTTP), an enzyme that facilitates VLDL production and its transport out of the liver, as well as phosphatidylethanolamine N-methyltransferase (PEMT), were significantly increased in livers of $Arg2^{-/-}$ mice when compared to WT mice (Fig. 2D). As the most prominent changes were observed in the DNL pathway we assessed protein levels of FAS and SCD-1 in liver lysates. In line with the aforementioned results we found a significant increase of both proteins in livers of $Arg2^{-/-}$ mice when compared to wild type mice (Fig. 2E). Taken together our findings suggest that arginase 2 deficiency is associated with the development of hepatic lipid accumulation and involves up-regulated DNL within the liver.

$Arg2^{-/-}$ mice show evidence of spontaneous inflammation with M1 polarized macrophages

Due to the potential role of arginase 2 deficiency in immune responses, we next focused on inflammatory signaling and found a marked infiltration of F4/80 positive cells in the livers of $Arg2^{-/-}$ when compared to WT mice (supplemental Fig. 1A). Analysis of mRNA levels revealed an increased expression predominantly in F4/80 (six-fold), in CD11c (three-fold), in IL1- β (six-fold), and in iNOS (four-fold) in $Arg2^{-/-}$ mice when compared to WT mice, indicating that predominantly M1 polarized proinflammatory macrophages are present in livers of $Arg2^{-/-}$ mice (supplemental Fig. 1B).

Liver-specific macrophage depletion results in reversal of hepatic steatosis and fibrosis in $Arg2^{-/-}$ mice

The findings of spontaneous hepatic steatosis and hepatic macrophage activation in the livers of $Arg2^{-/-}$ mice in conjunction with the fact that arginase 2 is normally poorly expressed in hepatocytes but present in monocyte/macrophages, lead us to explore the hypothesis that selective suppression of macrophages in the liver of $Arg2^{-/-}$ would result in the correction of the abnormal hepatic lipid accumulation found in these animals. To test this hypothesis we selectively depleted macrophages in the liver by intravenous injection of liposome-encapsulated clodronate (CLOD). During this process we did not observe differences in total body and liver weight between the CLOD-treated and PBS control treated $Arg2^{-/-}$ mice (Fig. 3A, B). Depletion of liver macrophages after four injections of liposome-encapsulated CLOD was confirmed by F4/80 immunohistochemistry (Fig. 3A). Oil-Red O staining of frozen liver sections revealed a notable attenuation of lipid deposition in the CLOD-treated mice compared to their PBS-treated counterparts (Fig. 3A). This corresponded to a decrease in liver triglyceride levels in the CLOD group compared to the PBS group (Fig. 3C). Furthermore, when expression levels of genes involved in lipid metabolism were examined by qRT-PCR, key enzymatic mediators of DNL and lipid export from the liver were found to be down-regulated in CLOD-treated mice when compared to PBS controls (Fig. 3D). Analysis of epididymal adipose tissue did not reveal significant differences in morphology and only showed a slight and non-significant reduction in macrophage infiltration (Fig. 3E, F). As expected, we found a depletion of macrophages in livers of WT mice injected with CLOD when compared to WT mice injected with PBS. While body weight, liver weight, epididymal adipose tissue weight, levels of liver triglycerides, and serum triglycerides did not differ in WT mice injected with CLOD when compared to mice injected with PBS, mRNA levels of F4/80 and arginase 2 were

significantly decreased in WT mice injected with CLOD (supplemental Fig. 2). Taken together, these findings strongly suggest that arginase 2-deficient macrophages play an important role in the accumulation of triglycerides in hepatocytes of *Arg2*^{-/-} mice.

Arginase 2 deficiency results in increase susceptibility to high fat diet induced liver injury

Our data revealed that arginase 2 deficiency results in spontaneous development of steatosis and inflammation. Next, to test our initial hypothesis that arginase 2 deficiency in mice would result in progression from isolated hepatic steatosis induced by high fat (HFAT) feeding to steatohepatitis, we examined the effects of short-term HFAT diet feeding on *Arg2*^{-/-} and WT animals (Fig. 4A). Gross examination of the livers from *Arg2*^{-/-} animals showed hepatomegaly that translated to an increased liver/body weight ratio compared to WT mice on the HFAT diet (Fig. 4B). *Arg2*^{-/-} mice on HFAT diet showed significant elevation of serum ALT levels that were 4–5-fold higher than the normal levels and significantly higher than those observed in the WT animals on the HFAT diet (Fig. 4B). Histological evaluation of liver sections revealed that, while WT mice developed as expected hepatocellular steatosis to a mild degree (in average 30%, grade 1), upon short-term HFAT feeding, hepatocytes of *Arg2*^{-/-} mice exhibited steatosis to a severe degree (average 85%, grade 3). Moreover, arginase 2 deficiency resulted in marked exacerbation of lobular inflammation with up to four spots of inflammatory foci per 20× magnification while livers of WT mice only exhibited one or two inflammatory foci per field of view (Fig. 4C, D). Hepatocellular ballooning was neither diagnosed in livers of *Arg2*^{-/-} mice nor WT mice. However, livers of *Arg2*^{-/-} showed numerous hepatocytes with multiple small droplet steatosis which made the distinction of balloon hepatocytes a significant challenge (Fig. 4C, D). This histological finding translated into increased mRNA levels of inflammatory marker (F4/80, TNF- α , and iNOS) in *Arg2*^{-/-} mice fed with high fat diet when compared to WT mice (Fig. 4E). Taken together our data identifies arginase 2 as a key mediator of increased susceptibility to high fat diet induced hepatic steatosis and liver damage.

DISCUSSION

The principal findings of this study relate to the role of arginase 2 in the development of hepatic steatosis and the progression to steatohepatitis. The results demonstrate that arginase 2 deficiency leads to spontaneous development of hepatic steatosis that is independent of weight gain and systemic metabolic changes and associated with increased hepatic de novo lipogenesis and liver Kupffer cell/macrophage activation towards a pro-inflammatory M1 phenotype. Selective depletion of Kupffer cells in *Arg2*^{-/-} mice results in protection from hepatic steatosis and liver inflammation in these animals, while arginase 2 deficiency favors progression from isolated hepatic steatosis induced by high fat feeding to steatohepatitis. This study identifies arginase 2 as a novel link between innate immune responses, hepatic lipid deposition, and liver injury.

Within the multi-hit model of NAFLD pathogenesis, growing evidence supports the concept that innate immune activation in the liver is a key “second hit” involved in disease progression to NASH. Activation of liver resident macrophages or Kupffer cells resulting in an increased production of pro-inflammatory cytokines as well as inducible nitric oxide

synthase (iNOS) has been proposed to play a pivotal role in this process [13]. Indeed, the liver microenvironment harbors numerous potential stimuli for macrophage activation, including both pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPS, respectively) [11]. Sources of PAMPs are structural motifs of proteins, lipids, and nucleic acids originating from invading microorganisms, while DAMPS are derived from injured, dying or malignantly transformed host cells [30]. Among the many exogenous danger molecules that are likely to play a role in liver disease, LPS has been extensively implicated in the pathogenesis of NAFLD. Evidence for the pivotal role of LPS in NAFLD development and progression includes increased circulating LPS levels in NAFLD patients, and increased sensitivity to LPS in experimental NAFLD models [31, 32]. In alcoholic liver disease, direct alcohol toxicity disrupts the intestinal epithelium impairing its barrier function, which leads to leaking of LPS from the gut lumen to the portal circulation [33]. In NAFLD, increased levels of and sensitivity to LPS have been proposed to result from impaired clearance by fatty hepatocytes and altered toll-like receptor 4 (TLR4) signaling, leading to insufficient control of danger recognition [30]. A recent report showed that patients with NAFLD do in fact, have both small intestinal bacterial overgrowth and increased intestinal permeability [34]. This increase in intestinal permeability may be a result of quantitative alterations in gut microbiota, hyperinsulinemia and increased circulating levels of inflammatory cytokines, or more likely a combination of all three [35]. Gut microbiota has also been shown to affect fat storage and energy metabolism via increased intestinal permeability leading to endotoxemia with subsequent triggering of inflammation and metabolic derangements [35]. For instance, microbiota has been shown to promote an increase in hepatic triglyceride content via upregulation of de novo fatty acid synthesis [36].

Mammals express two isoforms of arginase, an enzyme that competes with iNOS for its substrate, arginine, designated as types 1 and 2. Arginase 1 is mainly expressed in hepatocytes and it is the isoform that participates in the urea cycle [17]. Arginase 2 is poorly expressed in hepatocytes, and most highly expressed in kidney, prostate, and immune cells such as monocyte/ macrophages [17]. An imbalance in iNOS / arginase ratio in favor of the former have been postulated to play a crucial role in regulating immune responses and macrophage activation. Our data supports this concept by demonstrating that arginase 2 deficiency results in activation of Kupffer cells towards a classically “M1” activated, pro-inflammatory state. Unexpectedly, these changes in the livers of *Arg2*^{-/-} were associated with the spontaneous development of hepatic steatosis. The increase deposition of lipids in the liver was independent of changes in body weight and systemic metabolic changes. The interactions between fatty acid metabolism, insulin resistance, dyslipidemia and hepatic triglyceride content that contribute to hepatic steatosis in NAFLD are complex, and involve different organs and cell types [37]. Obese subjects with NAFLD show evidence of increased lipolysis in adipose tissue and subsequent increase in rate of free fatty acid (FFA) delivery to and uptake by liver [37].

Given our findings of unaltered circulating FFA and FFA uptake by liver in *Arg2*^{-/-} mice, impaired lipid metabolism resulting in de novo lipogenesis (DNL) within the liver was suspected as the mechanism leading to intrahepatic triglyceride accumulation in *Arg2*^{-/-}

mice. Indeed, increase in DNL is found in different experimental models of NAFLD as well as in patients with NAFLD [38]. The mechanisms involved in the activation of DNL in obese animals and humans remain incompletely understood, but increased plasma glucose and insulin levels in the context of a state of systemic insulin resistance is thought to be an important stimuli for DNL via activation of key transcription factors SREBP-1c and ChREBP [39]. In contrast with normal subjects in whom DNL contributes only marginally to total intrahepatic triglyceride production and FAs incorporated into VLDL, in human subjects with NAFLD, DNL accounts for as much as 23% of FAs within intrahepatic triglycerides and those secreted in VLDL [37]. The observed increase in DNL in *Arg2*^{-/-} mouse livers was not associated with evidence of insulin resistance which lead us to further explore the potential mechanism triggering de novo lipid synthesis in the livers of these mice.

Our data identify a novel link between Kupffer cell activation and hepatic steatosis by demonstrating that selective depletion of Kupffer cells in these mice resulted in a reversal of hepatic steatosis and normalization of the expression of genes involved in DNL. These results suggest that in the presence of arginase 2 deficiency, altered immune responses precede, and is responsible of lipid deposition in hepatocytes mainly by activation of de novo lipogenesis pathways in the liver.

The idea that steatosis is the necessary initial step preceding all other features of NAFLD, such as inflammation, has been recently challenged by studies demonstrating that inflammation can actually precede steatosis and even contribute to its development in both mouse models and in vitro studies. For instance, features of steatohepatitis examined after short-term high fat feeding (2, 4, 7 days) in APOE2 knock-in mice revealed prominent macrophage infiltration after just two days of HFD feeding, whereas lipid droplets were not visible until 4–7 days post-HFD feeding [40]. Furthermore, up-regulation of inflammatory genes preceded that of lipid metabolism genes, and expression of inflammatory genes was immediately reduced upon fenofibrate treatment, before that of lipid metabolism genes [40]. Our data supports this concept by identifying an experimental model in which altered immune activation appears as the initial mechanism leading to hepatic steatosis. These concepts have important implications for the understanding of NAFLD pathogenesis and potential development of novel treatment strategies for patients with this condition.

Finally our data demonstrates that arginase 2 deficiency increases the susceptibility of mice to fatty liver disease associated with DIO. Indeed, *Arg2*^{-/-} mice on a high fat diet showed marked elevation of serum liver enzymes and more severe lipid deposition and features of liver damage. A central question in human NAFLD is why patients with similar degrees of obesity, insulin resistance and other risk factors for lipid overloading of the liver develop different degrees of liver involvement. Our results identify arginase 2 deficiency as a novel deleterious trait contributing to disease progression and liver injury and warrants future investigations of arginase 2 status in human NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACADM	acyl-coenzyme A dehydrogenase
ALT	alanine aminotransferase
Arg1	arginase 1
Arg2	arginase 2
CD11c	cluster of differentiation 11c
CD36	cluster of differentiation 36 or fatty acid translocase
CD68	cluster of differentiation 68
CLOD	clodronate
CPT1	carnitine palmitoyl-transferase 1
DIO	diet induced obesity
DAMP	damage-associated molecular pattern
DNL	de novo lipogenesis
F4/80	mouse macrophage marker
FABP4	fatty acid binding protein 4
FAS	fatty acid synthase
FATP2/FATP5	fatty acid transport proteins 2/5
HFAT	high fat
IL1-beta	interleukin 1 beta
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
LXR-a	liver X receptor alpha
MTP	microsomal triglyceride transfer protein
NAFLD	Nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
ORO	Oil Red O
PAMP	pathogen-associated molecular pattern
PEMT	phosphatidylethanolamine N-methyltransferase

SCD-1	stearoyl-CoA desaturase 1
SREBP-1	sterol regulatory element binding protein 1c
TNF-α	tumor necrosis factor alpha
WT	wild type

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Figure 1. Spontaneous liver injury and hepatic steatosis is present in 14 weeks old $Arg2^{-/-}$ mice Body weight (A) along with several metabolic parameters – plasma glucose level, plasma insulin level, levels of free fatty acids in plasma, plasma triglycerides – are comparable in $Arg2^{-/-}$ mice and WT mice fed with normal chow (B). However, analysis of liver histology and assessment of hepatic steatosis with Oil Red O staining uncovered the development of liver injury and hepatic steatosis (C) (10 $\times$ and 40 $\times$). In line with this ALT, levels were significantly increased in $Arg2^{-/-}$ mice when compared to WT mice (D). The amount of

liver triglycerides was also increased in *Arg2*^{-/-} (*Arg2*^{-/-} 4.9 vs. WT 2.6 mg TG/g liver, $p < 0.05$, E). Mean $\pm$ S.E.M. are shown. * $p < 0.05$.

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Figure 2. Hepatic lipid metabolism is altered in *Arg2*^{-/-}

Among markers for fatty acid uptake and transport within the liver (CD36, fatty acid transport proteins (FATP) fatty acid binding proteins (FABPs) we found a significant increase in levels of FABP1 (A). Fatty acid synthesis and de novo lipogenesis (DNL) assessed by quantifying mRNA levels of fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase 1 (SCD-1), the transcription factor sterol regulatory element binding protein 1c (SREBP-1c), liver X receptor alpha (LXR-α), ATP citrate lyase (ACLY), and elongation of long chain fatty acids 2, 5 and 6 (Elovl2, 5, 6). Significant increases were

detected for mRNA levels of SREBP1c, SCD-1, LXR- α , ACLY, and Elovl2 in *Arg2*^{-/-} mice when compared to WT mice (B). Fatty acid oxidation, assessed by measuring mRNA levels of carnitine palmitoyl-transferase 1 (CPT-1), and acyl-coenzyme A dehydrogenase, C-4 to C-12 straight chain (ACADM) was not found to be different in *Arg2*^{-/-} and WT mice (C). Markers for triglyceride export - microsomal triglyceride transfer protein (MTTP) and phosphatidylethanolamine N-methyltransferase (PEMT) – were significantly increased in livers of *Arg2*^{-/-} when compared to WT mice (D). In assessing ARG2 levels in liver lysates we were able to document an almost complete absence of the protein in ARG2 knockout mice (E). Protein quantifications of FAS (273kDa) and SCD-1 (37kDa) revealed significantly increased levels in *Arg2*^{-/-} mice when compared to wild type mice (E). Mean $\pm$ S.E.M. are shown. *p<0.05. **p<0.01. ***p<0.001

Figure 3. Reversal of hepatic steatosis and inflammation in *Arg2*^{-/-} mice after liver-specific macrophage depletion

Selectively depleted macrophages in the liver by intravenous injection of liposome-encapsulated clodronate (CLOD) did not alter liver weight and metabolic status – assessed via plasma glucose levels – between the CLOD-treated and PBS control treated *Arg2*^{-/-} mice (A). Depletion of liver macrophages after four injections of liposome-encapsulated CLOD was confirmed by F4/80 immunohistochemistry (B). Oil-Red O staining revealed a notable attenuation of lipid deposition in the CLOD-treated mice compared to their PBS-

treated counterparts (C). This corresponded to a decrease in liver triglyceride levels in the CLOD group compared to the PBS group (C). Expression levels of genes involved in DNL and lipid export from the liver were found to be reduced in CLOD-treated mouse livers compared to PBS controls (D). No differences were observed in gross morphology of adipose tissue samples of mice treated with CLOD (E). Macrophage infiltration assessed via F4/80 immunohistochemistry only showed a slight reduction in mice treated with CLOD when compared to mice injected with PBS (E, F). Microphotographs are displayed in 10× and 40× magnifications. Mean ± S.E.M. are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. HFAT feeding exacerbates the high fat diet induced liver injury in *Arg2*^{-/-} mice
 Within a short term feeding period with high fat (HFAT) diet for 6 weeks, *Arg2*^{-/-} and WT animals gained significant weight (A). Livers of *Arg2*^{-/-} mice weighed significantly more than those of WT mice after 6 weeks of high fat diet and ALT serum levels were significantly elevated in *Arg2*^{-/-} mice on HFAT diet (B). Histological analysis of liver samples of revealed that *Arg2*^{-/-} mice develop more steatosis than WT mice (C). More importantly, *Arg2*^{-/-} mice showed increased lobular increased lobular inflammation which translated into an increase in the NAFLD-activity score (NAS). In line with this, global

marker for macrophages (F4/80), as well as, markers for inflammatory macrophage polarization (TNF- α and iNOS) were increased in *Arg2*^{-/-} mice when compared to WT mice on HFAT diet (E). Microphotographs are displayed in 10 $\times$ and 40 $\times$ magnifications. Mean $\pm$ S.E.M. are shown. *p<0.05, **p<0.01.

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