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A cell-type-specific role for murine Commd1 in liver inflammation

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Abstract

The transcription factor NF- κ B plays a critical role in the inflammatory response and it has been implicated in various diseases, including non-alcoholic fatty liver disease (NAFLD). Although transient NF- κ B activation may protect tissues from stress, a prolonged NF- κ B activation can have a detrimental effect on tissue homeostasis and therefore accurate termination is crucial. Copper Metabolism MURR1 Domain-containing 1 (COMMD1), a protein with functions in multiple pathways, has been shown to suppress NF- κ B activity. However, its action in controlling liver inflammation has not yet been investigated. To determine the cell-type-specific contribution of Commd1 to liver inflammation, we used hepatocyte and myeloid-specific Commd1-deficient mice. We also used a mouse model of NAFLD to study low-grade chronic liver inflammation: we fed the mice a high fat, high cholesterol (HFC) diet, which results in hepatic lipid accumulation accompanied by liver inflammation. Depletion of hepatocyte Commd1 resulted in elevated levels of the NF- κ B transactivation subunit p65 (RelA) but, surprisingly, the level of liver inflammation was not aggravated. In contrast, deficiency of myeloid Commd1 exacerbated diet-induced liver inflammation. Unexpectedly we observed that hepatic and myeloid Commd1 deficiency in the mice both augmented hepatic lipid accumulation. The elevated levels of proinflammatory cytokines in myeloid Commd1-deficient mice might be responsible for the increased level of steatosis. This increase was not seen in hepatocyte Commd1-deficient mice, in which increased

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lipid accumulation appeared to be independent of inflammation. Our mouse models demonstrate a cell-type-specific role for *Commd1* in suppressing liver inflammation and in the progression of NAFLD.

Keywords

COMMD1; inflammation; NAFLD; macrophages; NF- κ B inhibitor

1. Introduction

The Copper Metabolism Murr1 Domain-containing protein 1 (COMMD1) is the founder member of a relatively new family of proteins, the COMMD family [1]. This protein family is distinguished by a unique motif called the COMM domain, located at their carboxy-terminus. Recent studies have demonstrated that COMMD1 acts in a wide variety of cellular processes, including hepatic copper transport [2] [3], hypoxia response [4] [5] [6], sodium, potassium and chloride transport [7] [8] [9] [10], and in nuclear factor kappa B (NF- κ B) signaling [11]. We recently confirmed its role in hepatic copper homeostasis in liver-specific *Commd1* knockout mice [12]. On depletion of *Commd1* in hepatocytes, mice become susceptible to hepatic copper accumulation [12], similar to dogs carrying a homozygous *COMMD1* loss-of-function mutation [2]. Notwithstanding its role in copper transport, the biological role of COMMD1 in NF- κ B signaling in the liver and in inflammatory liver diseases has not yet been defined.

The NF- κ B family of transcription factors plays a key role in the inflammatory responses. The family consists of five members, of which p65 (RelA) and p50/p105 (NF- κ B1) compose the canonical NF- κ B pathway. The p65/p50 heterodimer is sequestered in the cytoplasm by the inhibitory I κ B proteins. Activation of the canonical NF- κ B pathway via the kinase complex IKK results in translocation of p65/p50 dimer to the nucleus for transcriptional activation of its target genes. COMMD1 has been shown to terminate NF- κ B activity by acting as a scaffold protein in the E3 ubiquitin ligase complex (ECS^{SOCS1}) [1] [13]. ECS^{SOCS1} promotes ubiquitination and subsequent proteasomal degradation of p65 and destabilizes the interaction between p65 and chromatin. Hence, depletion of COMMD1 results in elevated p65 levels and subsequently increased NF- κ B activity [1] [13] [14].

The NF- κ B signaling pathway has a remarkable physiological function in several liver diseases, including non-alcoholic fatty liver disease (NAFLD) [15]. NAFLD consists of a wide spectrum of pathologies, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), and can even progress to liver fibrosis and cirrhosis, and in some cases to hepatocellular carcinoma (HCC) [16]. The progression to the severe stages of NAFLD, which are related to a poor prognosis, is thought to be driven by inflammation, including the expression of the NF- κ B-mediated cytokines Il-6 and Tnf- α [16] [17] [18]. These proinflammatory cytokines are mainly secreted by activated Kupffer cells, the resident liver macrophages, and they promote the progression of NAFLD towards NASH [19] [20]. In addition, the NF- κ B signaling pathway in hepatocytes also plays a role in NAFLD progression, as hepatocyte-specific depletion of NEMO, the regulatory subunit of the IKK complex, results in chronic steatohepatitis and eventually leads to the formation of liver

tumors [21]. Together these findings underscore the pivotal role of the NF- κ B signaling pathway in health and disease, but the contribution of COMMD1 in hepatocyte NF- κ B signaling and in inflammatory liver diseases still remains elusive. To determine the cell-type-specific role of COMMD1 in liver inflammation, we used hepatic and myeloid-specific *Comm1*-deficient mice and a second mouse model of NAFLD for low-grade, chronic liver inflammation. In these different mouse models, we studied the level of diet-induced liver inflammation and the progression of hepatic steatosis.

2. Materials and Methods

2.1 Animals

Conditional hepatocyte-specific (*Comm1*^{Hep}) [12] and conditional myeloid-specific knockout mice (*Comm1*^{Mye}) were obtained by crossing *Comm1*^{loxP/loxP} mice (here referred to as wild type (WT) mice) with Albumin-Cre [22] or LysM-Cre [23] transgenic mice, respectively. Both *Comm1*^{Hep} and *Comm1*^{Mye} mice were backcrossed in a C57BL/6J background for more than 8 generations. *Comm1*^{loxP/loxP} littermate mice (WT) served as controls for *Comm1*^{Hep} and *Comm1*^{Mye} mice. *p55*^{ns/ns}; *Comm1*^{Hep} were obtained by crossing *p55*^{ns/ns} [24] with *Comm1*^{Hep} mice. All the experimental mice were males and were housed individually. They were fed *ad libitum* with either standard rodent chow diet (RMH-B, AB Diets, Woerden, the Netherlands), or, starting at 8–10 weeks of age, a high-fat, high-cholesterol (HFC) diet (45% calories from butter fat) containing 0.2% cholesterol (SAFE Diets) for a period of 12 weeks. *p55*^{ns/ns}; *Comm1*^{Hep} and *p55*^{ns/ns} mice were fed only a chow diet and were sacrificed at the age of 20 weeks. All animals were sacrificed following a 4-hour morning fasting period. Body weight and liver weight measurements were recorded. Collected tissues were snap-frozen in liquid nitrogen and blood was collected by means of heart puncture in K3EDTA-coated MiniCollect® tubes (#450476, Greiner Bio-One, Alphen a/d Rijn, the Netherlands). The right hepatic lobe was used for gene expression, immunoblot and histological analysis. Plasma was separated by centrifuging at 3000 rpm for 10 min. at 4°C. All animal-related studies were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, the Netherlands).

2.2 Liver nuclear and cytosolic fraction isolation, DNA binding ELISA

Fractionation was performed on fresh, ice-cold, mouse liver samples, using the Nuclear Extract Kit (#40010, Active Motif, La Hulpe, Belgium) according to the manufacturer's instructions. To study the activity of NF- κ B in fresh livers, the DNA binding of p65 was assessed using the TransAM NF- κ B p65 ELISA kit (#40096, Active Motif, La Hulpe, Belgium) according to the manufacturer's instructions.

2.3 Isolation of bone marrow cells and peritoneal macrophages

Bone marrow cells isolated from either WT or *Comm1*^{Mye} mice were cultured and differentiated into macrophages, as described previously [25]. Peritoneal macrophages were isolated 3 days after injection of 4% thioglycolate in the peritoneal cavity of either WT or *Comm1*^{Mye} mice.

2.4 Immunoblot analysis

Tissues were homogenized in NP40 buffer [0.1% Nonidet P-40 (NP-40), 0.4 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA] supplemented with protease and phosphatase inhibitors and 30 µg of protein was loaded per gel lane. Samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Amersham™ Hybond™-P PVDF Transfer Membrane (#RPN303F, GE Healthcare, Diegem, Belgium). Bands were visualized using ChemiDoc™ XRS+ System (Bio-Rad Laboratories BV, Veenendaal, the Netherlands).

2.5 Liver lipid extraction

15% (w/v) liver homogenates were prepared in 1x PBS and lipid extraction was performed using the Bligh & Dyer method [26]. Samples were analyzed for cholesterol and triglyceride content.

2.6 Cholesterol and triglyceride analysis in plasma and liver lipid samples

Total cholesterol (TC) levels were determined using a colorimetric assay (11489232, Roche Molecular Biochemicals) with cholesterol standard FS (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) as a reference. Triglyceride (TG) levels were determined using Trig/GB kit (1187771, Roche Molecular Biochemicals) with Roche Precimat Glycerol standard (16658800) as a reference.

2.6 Antibodies

In these experimental procedures we used the following antibodies: rabbit polyclonal antibody against COMMD1 (11938-1-AP, Proteintech Group, USA), mouse anti-β-Actin (A5441, Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands), rabbit anti-Tubulin (AB4047, Abcam, Cambridge, UK), rabbit anti-Lamin A/C (2032, Cell Signaling Technology Europe, B.V., Leiden, the Netherlands), rabbit anti-p65 (4764, Cell Signaling Technology, Europe, B.V.), rabbit anti-IκBα (sc-371, Santa Cruz Biotechnology Inc., Heidelberg, Germany), rabbit anti-Cd68 (#137002, Biolegio, Nijmegen, the Netherlands), rabbit anti-F4/80 (#101201, Biolegio, Nijmegen, the Netherlands), goat anti-rabbit IgG (H + L)-HRP Conjugate (170-6515, Bio-Rad Laboratories BV, Veenendaal, the Netherlands), goat anti-mouse IgG (H + L)-HRP Conjugate (170-6516, Bio-Rad Laboratories BV).

2.7 Liver histology

Paraffin-embedded liver sections (4 µm) were stained with Hematoxylin & Eosin (H&E). Snap-frozen liver sections (5 µm) were stained using Oil Red O (ORO) or antibodies against Cd68. F4/80 staining was performed on either paraffin-embedded or snap-frozen liver sections. Scoring of steatosis and lobular inflammation was performed in an unbiased manner by an experienced, certified veterinary pathologist using a method described previously [27].

2.8 Gene expression analysis

Pieces of murine liver of approximately 100 mg were homogenized in 1 ml QIAzol Lysis Reagent (Qiagen, Venlo, the Netherlands). Total RNA was isolated by chloroform

extraction. Isopropanol-precipitated and ethanol-washed RNA pellets were dissolved in RNase/DNase free water. 1 µg of RNA was used to prepare cDNA with the Quantitect Reverse Transcription Kit (Qiagen, Venlo, the Netherlands) according to the protocol provided by the manufacturer. 20 ng cDNA was used for subsequent quantitative real-time PCR (qRT-PCR) analysis using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories BV,) and 7900HT Fast Real-Time PCR System (Applied Biosystems). The following PCR program was used: 50°C/2 min., 95°C/10 min., 40 cycles of 95°C/15 sec and 60°C/1 min. Expression data were analyzed using SDS 2.3 software (Applied Biosystems) and the standard curve method of calculation. Mouse *Cyclophilin A* was used as an internal control gene. The primer sequences we used are listed in Table S1.

2.9 Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was performed using Prism 5.00 for Windows (GraphPad Software, CA, USA) and the unpaired Student's *t* test. Results of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1 Hepatic depletion of *Commd1* results in increased levels of NF-κB subunit p65

To elucidate the role of hepatic *Commd1* in NF-κB signaling and inflammation *in vivo*, we depleted *Commd1* in hepatocytes (*Commd1*^{Hep}) by crossing *Commd1*^{loxP/loxP} mice with Alb-Cre transgenic mice, mice expressing Cre-recombinase in adult hepatocytes [12]. *Commd1*^{Hep} mice showed marked reduction in hepatic *Commd1* levels, however some residual amount of *Commd1* was detected (Fig. 1A), which is likely due to the expression of *Commd1* in nonparenchymal cells, as approximately 80% of an adult liver genome exists in hepatocytes, the rest is located in endothelial, stellate or Kupffer cells [28].

Since various cellular models demonstrated that down-regulation of COMMD1 results in elevated p65 levels and subsequently increased NF-κB activity [1] [13], we first assessed the levels of p65 in nuclear and cytosolic fractions of livers from WT (n=6) and *Commd1*^{Hep} mice (n=6–8) (Fig. 1A). We observed that *Commd1*^{Hep} mice showed clearly higher protein levels of p65 in both the cytosolic and nuclear fractions of livers compared with WT mice.

Next, we determined whether the rise in protein p65 levels was caused by an increase in *p65* mRNA levels (Fig. 1B). We detected no difference in hepatic *p65* gene expression between *Commd1*^{Hep} mice and WT littermates, excluding the possibility that the increase in p65 protein levels was due to alterations in transcriptional regulation. In line with previous *in vitro* studies [1] [13], these data suggest that *Commd1* depletion results in an increased protein stability of p65 in hepatocytes.

3.2 Hepatic *Commd1* depletion aggravates steatosis, but not inflammation

Since NF-κB-mediated inflammation is associated with the progression of NAFLD towards a more severe NASH phenotype [29] [30] [31], we investigated the consequences of elevated p65 levels in hepatic *Commd1*-deficient mice on inflammation in a mouse model of NAFLD induced by an HFC diet. After 12 weeks of HFC feeding, we saw no differences in

body and liver weight between *Commd1*^{Hep} and WT mice (Fig. 2A). In addition, no liver damage was observed, as the plasma levels of the liver enzymes ALT and AST were not markedly increased (data not shown). Surprisingly however, total hepatic cholesterol and triglyceride levels were significantly increased in the *Commd1*^{Hep} mice following 12 weeks of HFC diet (Fig. 2B). This observation was supported by histological analysis: hematoxylin and eosin (H&E) staining demonstrated an increase in lipid deposits in the livers of *Commd1*^{Hep} mice (Fig. 2C, D), as confirmed by ORO staining (Fig. 2C). These differences were not seen in chow-fed animals. Histologically, HFC-feeding markedly increased the level of lobular inflammation in both WT and *Commd1*^{Hep} mice, but no alterations between the genotypes were seen (Fig. 2E).

In order to investigate the effect of *Commd1* loss in hepatocytes on inflammation in greater detail, we performed immunohistochemical stainings. Immunostaining for the macrophage markers [32] Cd68 (a marker of activated macrophages [33]) and F4/80 (marker of mature macrophages, highly expressed by Kupffer cells [33]) showed increased infiltration of macrophages in the livers on HFC feeding (Fig. 3A), but we saw no differences between *Commd1*^{Hep} and WT mice. Expression analysis of *Cd68* and *F4/80*, together with *Cd11b*, a migratory marker of blood-derived monocytes [34], confirmed the immunohistochemical results (Fig. 3B).

Next, hepatic mRNA levels of a number of NF- κ B target genes were determined (Fig. 3C). A significant increase in the expression of the proinflammatory genes: *Tnfa*, *Il-1 α* , *Il-1 β* and *Mcp1*, and the NF- κ B target genes: *Icam*, and *Tnfaip3* (*A20*) was detected following 12 weeks of HFC feeding, but we saw no differences between *Commd1*^{Hep} and WT mice, corroborating the histological analysis. In addition, no substantial difference in the expression of other NF- κ B (Fig. S1A) or *Commd* genes was seen (Fig. S1B). Altogether, hepatic deficiency of *Commd1* exacerbated HFC diet-induced steatosis, but not liver inflammation.

Since *Commd1* is involved in multiple physiological processes [3] [11] [35], it is possible that dietary intervention in combination with *Commd1* deficiency affects additional pathways that modulate diet-induced liver inflammation, independent of its role in NF- κ B signaling, leading to the observed results. Therefore, we decided to use a genetic approach to further evaluate the role of hepatocyte *Commd1* in NF- κ B-mediated liver inflammation. We crossed *Commd1*^{Hep} mice on a *p55*^{ns/ns} genetic background (*p55*^{ns/ns}; *Commd1*^{Hep}). The *p55*^{ns/ns} mice are homozygous for a mutation in the gene encoding the tumor necrosis factor receptor 1 (Tnfr1). This mutation results in impaired shedding of the Tnfr1 from the cell surface, resulting in increased activation of NF- κ B and chronic, low-grade inflammation in the liver [24] [36]. The *p55*^{ns/ns}; *Commd1*^{Hep} mice were born without any overt phenotype and in the expected Mendelian ratios. No differences in body and liver weight were observed (Fig. 4A, B). In line with the phenotype of *Commd1*^{Hep} mice, hepatic *Commd1* ablation in *p55*^{ns/ns} mice also resulted in elevated levels of p65 (Fig. 4C), with no alteration in *p65* mRNA levels (Fig. 4D). Furthermore, similar to what we and others have previously shown [24] [36], *p55*^{ns/ns} mice display a significant increase in the number of inflammatory foci within hepatic lobules (Fig. 4E). However, we saw no clear differences in the number of inflammatory foci between *p55*^{ns/ns} (n=6) and *p55*^{ns/ns};

Commd1^{Hep} mice (n=7) (Fig. 4E). This observation was corroborated by the fact that the gene expression of proinflammatory markers and cytokines was not affected by *Commd1* deficiency (Fig. 4F). Only *Il-1 α* mRNA levels were significantly increased, but the level of induction was rather mild. In addition to the NF- κ B signaling pathway, TNF- α also activates apoptotic pathways [37] [38], and since NF- κ B drives the expression of anti-apoptotic genes, we also looked at the mRNA levels of anti-apoptotic genes mediated by NF- κ B (Fig. 4G). However, we saw no differences between the two groups (Fig. 4G).

Altogether, using two independent but complementary approaches, we showed that depletion of *Commd1* in hepatocytes leads to elevated levels of the NF- κ B subunit p65, both in the nucleus and cytoplasm, but that it does not affect the level of liver inflammation induced by HFC-feeding nor in Tnf-mediated chronic hepatitis.

3.3 Steatosis and inflammation are exacerbated in myeloid-deficient *Commd1* mice

In addition to hepatocytes, myeloid cells (in particular macrophages) also play a crucial role in NF- κ B-mediated liver inflammation and in the progression of NAFLD [39]. We therefore assessed the role of myeloid *Commd1* in liver inflammation during the development of steatohepatitis. We crossed mice carrying floxed conditional *Commd1* alleles with *LysM-Cre* transgenic mice [23] to specifically ablate *Commd1* in the myeloid lineage (Fig. S2A,B) [40]. We fed WT (n=6–7) and *Commd1*^{Mye} mice (n=6–7) either chow or HFC diet for 12 weeks. *Commd1* deficiency in myeloid cells did not lead to differences in body and liver weight, neither in chow- nor HFC-fed mice (Fig. 5A). The plasma levels of the liver enzymes ALT and AST were also not noticeably elevated (data not shown). However, HFC-fed *Commd1*^{Mye} mice showed a significant increase in liver triglyceride levels compared to WT mice (Fig. 5B). H&E staining of the livers corroborated the exacerbated liver steatosis in *Commd1*^{Mye} mice, and was further confirmed by ORO staining (Fig. 5C, D).

In addition to the elevated hepatic fat deposits, histological scoring also revealed an increase in hepatic inflammation (Fig. 5E). The microscopic appearance of the livers showed inflammatory foci widespread in the hepatic tissue. We therefore investigated the effect of myeloid *Commd1* depletion on liver inflammation in more detail. We stained liver sections of *Commd1*^{Mye} and WT mice for Cd68 and F4/80 (Fig. 6A). Histological scoring showed an increase in the number of inflammatory foci in *Commd1*^{Mye} mice following 12 weeks of HFC feeding. Moreover, this observation was confirmed by mRNA expression analysis (Fig. 6B). In addition, we analyzed the expression of various proinflammatory cytokines regulated by NF- κ B, such as *Tnf*, *Mcp-1*, *Ccl5* and *Icam* (Fig. 6C). Dietary intervention markedly induced the expression of proinflammatory markers in both groups. Compared to WT mice, *Commd1*^{Mye} mice showed a significant increase in mRNA expression of most of the proinflammatory markers studied, except for *Cd11b* and *Ccl5*, which both showed a trend towards elevated expression (Fig. 6B, C). In conclusion, depletion of *Commd1* in myeloid cells leads not only to increased liver inflammation, but also exacerbates the progression of steatosis upon 12 weeks of HFC feeding.

4. Discussion

NF- κ B signaling is an essential pathway in the progression of many inflammatory diseases, including NAFLD [41] [42]. It is therefore crucial to identify the genes and mechanisms regulating the NF- κ B pathway, and these might lead to novel therapeutic strategies to treat NAFLD. COMMD1, a pleiotropic protein, is involved in various pathways including NF- κ B signaling [1] [11]. Here we evaluated the extent Commd1 deficiency in either hepatocytes or macrophages contributes to liver inflammation and progression of NAFLD in mice. On the one hand we showed that Commd1 has a cell-type-specific role in controlling liver inflammation in NAFLD, since myeloid Commd1 deficiency, but not hepatocyte-specific deletion, augmented the inflammatory tone of the disease. On the other hand, we saw that depletion of Commd1 in either cell type exacerbated diet-induced hepatic lipid accumulation.

Ablation of Commd1 in the myeloid lineage caused increased diet-induced steatosis and liver inflammation concomitant with the elevated expression of several inflammatory cytokines, in particular *Tnfa*. Kupffer cells are the main source of hepatic TNF α , which has been shown to be an essential cytokine in the progression of NAFLD [39]. Blocking the Tnf α signaling pathway by deletion of either the *Tnfr1* or *Tnfa* ameliorates NAFLD in mice [20] [43] [44]. In addition, leptin-deficient (*Ob/Ob*) mice treated with anti-TNF α antibodies show a reduced level of liver steatosis [45] [46] [47]. The increased lipid accumulation observed in HFC-fed *Commd1*^{Mye} mice might therefore be explained by the elevated *Tnfa* expression in these mice. Our observation of a higher inflammatory tone in the liver of HFC-fed *Commd1*^{Mye} mice is in line with our recent study [40], in which we showed that myeloid depletion of Commd1 exacerbates dextran sodium sulfate (DSS)-induced colitis and increases the susceptibility to sepsis because it invokes a stronger inflammatory response. Furthermore, Commd1 deficiency in bone-marrow derived myeloid cells selectively altered the expression of LPS-mediated genes, including a subset of genes involved in the immune response, and genes directly regulated by NF- κ B [40]. However, these expression data also demonstrated that in addition to NF- κ B, myeloid Commd1 also mediates other pathways activated by LPS, either directly or indirectly [40]. In addition, the intestinal epithelial-deficient Commd1 mice do not show increased inflammation or any sensitivity difference in DSS-induced colitis, resembling some aspects of the hepatic-specific deficiency that we present here.

Despite the elevated levels of cytosolic and nuclear p65 (Fig. 1A), Commd1 deficiency in hepatocytes did not affect the level of liver inflammation in either NAFLD (Fig. 3) or in mice with low-grade liver inflammation due to a mutation in *Tnfr1* [24] [36]. Nonetheless, the increase in p65 levels is in line with previous *in vitro* studies [1] [13], which demonstrated that COMMD1 promotes the ubiquitin-mediated proteolysis of p65. Insufficiency of COMMD1 in U2OS cells [13] or loss of p65-COMMD1 interaction [14] increased the steady-state and the protein stability of p65, respectively. Together with the unchanged mRNA levels of p65 (Fig. 1B), these data suggest that the elevated p65 levels in Commd1-deficient hepatocytes may result from an increased protein stability of p65 caused by reduced p65 ubiquitination. Nevertheless, independent of the level of hepatocyte p65, the activity of NF- κ B is not changed upon depletion of Commd1 (Fig. 3C). A DNA-binding

ELISA assay to assess the activity of NF- κ B supported this observation. Although LPS injection itself significantly increased the activity of NF- κ B in the livers of WT and *Commd1*^{Hep} mice, *Commd1* deficiency did not affect the level of NF- κ B binding to DNA neither after PBS nor LPS (Fig. S3). The level of NF- κ B activity is tightly titrated through various mechanisms [48] [49] [50] [51] [52] [53] and numerous proteins controlling NF- κ B signaling have been identified [11]; we therefore speculate that the effect of *Commd1* loss is compensated by another mechanism to restore a basal NF- κ B activity. We excluded the contribution of the well-known NF- κ B inhibitors, I κ B α Nfkb α and A20 (Tnfaip3) [11]. NF- κ B drives the expression of both genes, but the mRNA levels of *I κ B α Nfkb α* and *A20* (*Tnfaip3*) in *Commd1*^{Hep} livers of chow- and HFC-fed mice were not altered compared to WT mice (Fig. 3C). In line with this observation, we saw no difference in I κ B α Nfkb α protein levels in *p55*^{ns/ ns}; *Commd1*^{Hep} mice (Fig. 4C). In addition, we saw no marked differences in the expression of other COMMD genes, a family of proteins, which have the ability to inhibit NF- κ B activity [1] [11]. This suggests that there is another homeostatic mechanism that prevents uncontrolled NF- κ B activity in *Commd1*-deficient hepatocytes, which requires further studies to identify the mechanism and understand what is happening.

Despite the lack of a higher inflammatory response, *Commd1*^{Hep} mice fed a HFC-diet surprisingly showed elevated levels of liver cholesterol and triglycerides (TG) compared to WT littermates (Fig. 2B). Supported by histological analysis, these data indicate that hepatic *Commd1* deficiency aggravates steatosis. Although COMMD1 has been linked to the regulation of biliary copper excretion and may regulate trafficking of various transporters [2] [9] [54], including ATP7B, a P-type ATPase which mediates copper excretion into the bile [55], we could not observe any marked changes in the biliary cholesterol excretion determined by the *in vivo* Transintestinal Cholesterol Excretion (TICE) experiment [56] (data not shown). Because we did not observe any marked changes in the mRNA levels of various genes involved in lipid uptake, synthesis and excretion (data not shown), a clear explanation for this observation is still missing. However, as COMMD1 is associated with the intracellular trafficking of various proteins and is localized to vesicles (reviewed in [3]), we speculate that COMMD1 acts as an adaptor protein in sorting/fusion of vesicles, a process that is also involved in autophagy. Recent studies demonstrated that inhibition of macroautophagy is associated with accumulation of TG and cholesterol in lipid droplets [57] [58]. It would therefore be of interest to further investigate the hepatic function of COMMD1, and to determine which kind of vesicles COMMD1 is localized to. Although COMMD1 partially co-localizes to endosomal and lysosomal markers (reviewed in [3]), COMMD1-associated vesicles are still not fully characterized. Based on its pleiotropic function, it is highly possible that COMMD1 is not only involved in biliary copper excretion, but requires further substantial investigation.

In conclusion, in this study we demonstrate that *Commd1* represses the level of inflammation in NAFLD in a cell-type-dependent manner. Although hepatocyte *Commd1* does not play a major role in liver inflammation, our data indicate that it does have a protective role in slowing the progression of steatosis in mice. Furthermore, our current knowledge advocates that its repressive action on inflammation is restricted to myeloid cells and this seems to be a general phenomenon in various disease models [40]. The mechanism

by which myeloid COMMD1 restrains inflammation might therefore be an interesting target for developing new treatment strategies for inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Atp7B	ATPase, Cu ²⁺ transporting, beta polypeptide
TNFAIP3 (A20)	TNF α -Induced Protein 3
COMMD	Copper Metabolism gene MURR1 Domain -containing protein
ELISA	Enzyme-Linked Immunosorbent Assay
H&E	Hematoxylin and Eosin
HCC	Hepatocellular Carcinoma
HFC	High Fat, High Cholesterol
HFD	High Fat Diet
HIF	Hypoxia Inducible Factor
Icam	Intercellular Adhesion Molecule
IκB	Inhibitor of κ B
IL	Interleukin
LPS	Lipopolysaccharide
LysM	Lysozyme M
Mcp-1	Monocyte Chemoattractant Protein 1
MEFs	Mouse Embryonic Fibroblasts
NAFLD	Non-Alcoholic Fatty-Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NF-κB	Nuclear Factor κ B
ORO	Oil Red O
TICE	Trans-Intestinal Cholesterol Excretion
TNF	Tumor Necrosis Factor
TNFR1	Tumor Necrosis Factor Receptor 1

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Highlights

- COMMD1 is a negative modulator of inflammation
- Hepatic inflammation is suppressed by myeloid but not hepatocyte Commd1
- Hepatic COMMD1 mediates the level of p65 in hepatocytes, but not its activity

Depletion of hepatocyte or myeloid Commd1 both aggravates diet-induced steatosis

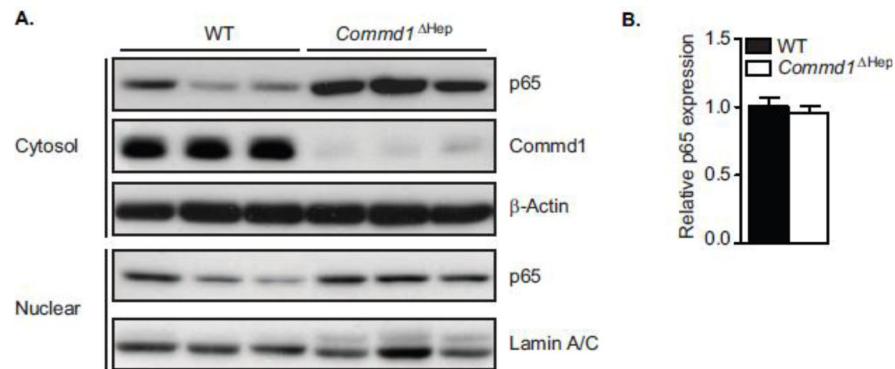


Figure 1. Commd1 mediates the levels of cytosolic and nuclear p65 in hepatocytes

(A) Fresh livers from chow-fed WT and *Commd1*^{Hep} mice were used to isolate nuclear and cytosolic fractions, then p65 levels were determined by immunoblot analysis. Three representative mice per group are shown. (B) Relative mRNA expression of *p65* in livers of WT and *Commd1*^{Hep} mice, as determined by quantitative RT-PCR. All values per group are shown as mean ± SEM.

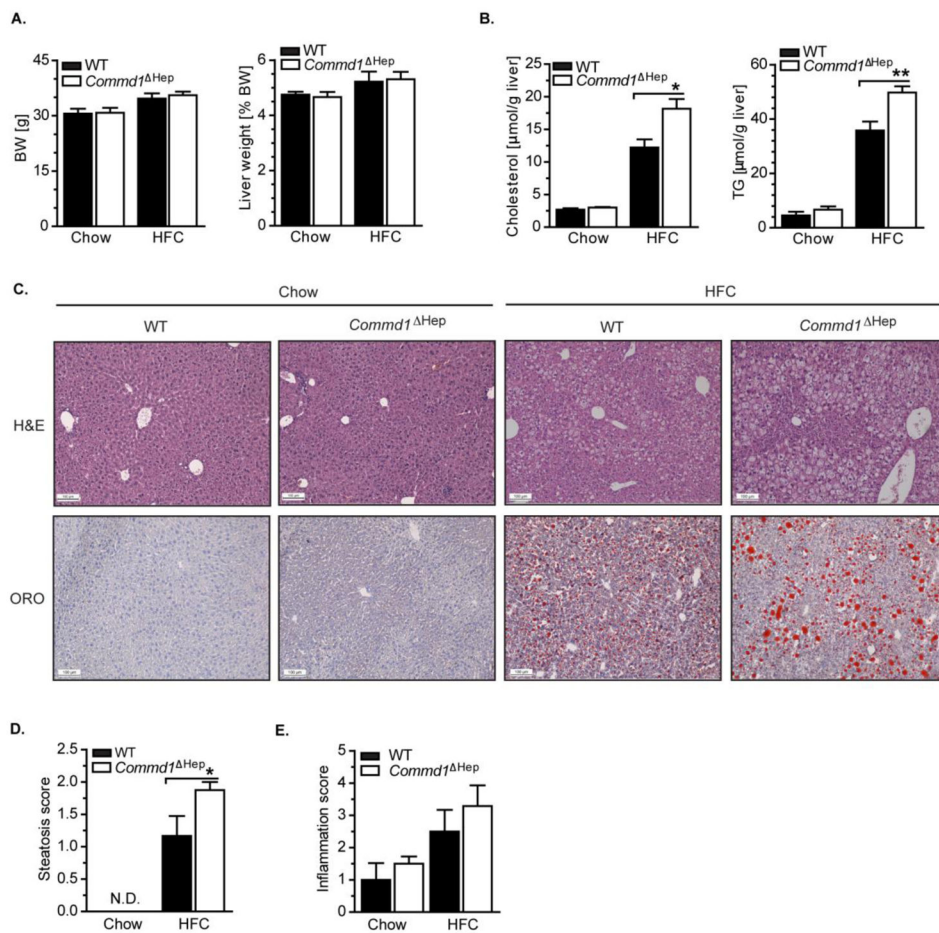


Figure 2. Hepatic *Commd1* deficiency aggravates lipid accumulation in HFC-fed mice (A) Body weight (BW) and liver weight, represented as % of the BW, of WT and *Commd1*^{Hep} mice after 12 weeks on HFC diet and of control chow-fed groups. (B) Hepatic total cholesterol and triglyceride levels. Liver lipids were extracted from snap-frozen mouse livers using the Blich-Dyer method for lipid extraction and analyzed with a colorimetric assay. (C) H&E and ORO staining of hepatic tissue from 4-hour fasted chow- and HFC-fed mice. H&E staining was performed on paraffin-embedded samples and ORO staining on snap-frozen hepatic cryo-sections. Representative images per group are shown. Scale bars represent 100 μm. (D) Histological evaluation of liver steatosis. Steatosis was not present in chow-fed mice (N.D. = not detected). (E) Histological evaluation of inflammation. Inflammation score was based on the number of inflammatory foci per five random fields at 200x. All values per group are shown as mean ± SEM. Statistical significance was determined versus WT control mice: *P<0.05, **P<0.01.

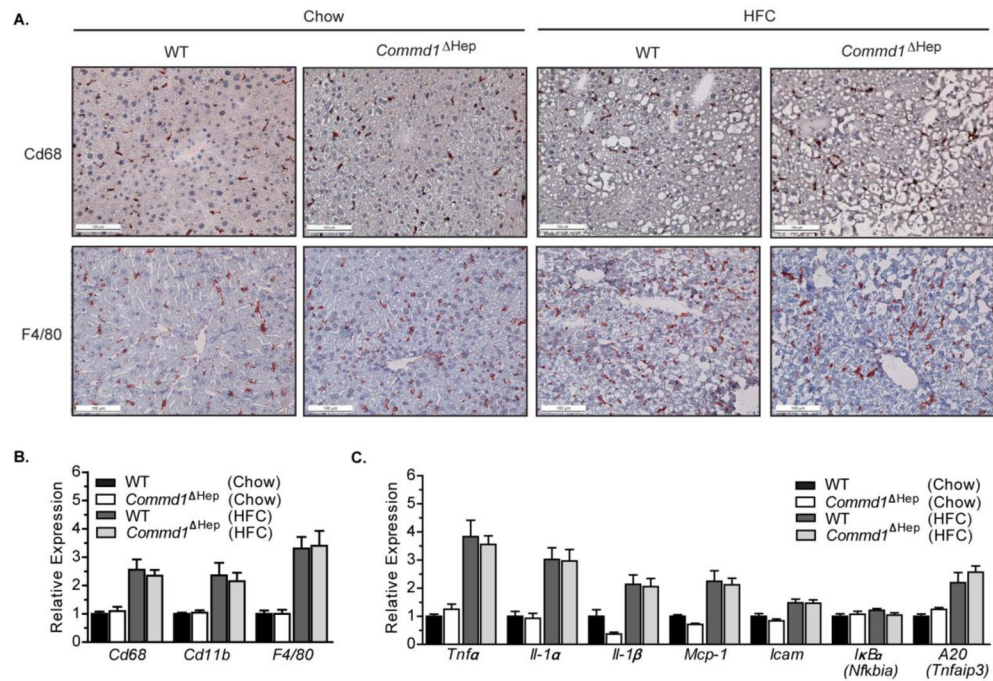


Figure 3. Depletion of hepatocyte *Commd1* has no effect on HFC-diet-induced liver inflammation

(A) Immunostaining of WT and *Commd1*^{Hep} livers. Snap-frozen samples were stained for the macrophage markers Cd68 and F4/80. Representative images per group are shown. Scale bars represent 100 μm. Relative liver mRNA expression of (B) the macrophage and monocyte markers *Cd68*, *Cd11b* and *F4/80*, and (C) proinflammatory cytokines and NF-κB target genes, *Tnfa*, *Il-1α*, *Il-1β*, *Mcp-1*, *Icam*, *IκBβ* (*Nfκbia*), and *A20* (*Tnfaip3*). All values per group are shown as mean ± SEM.

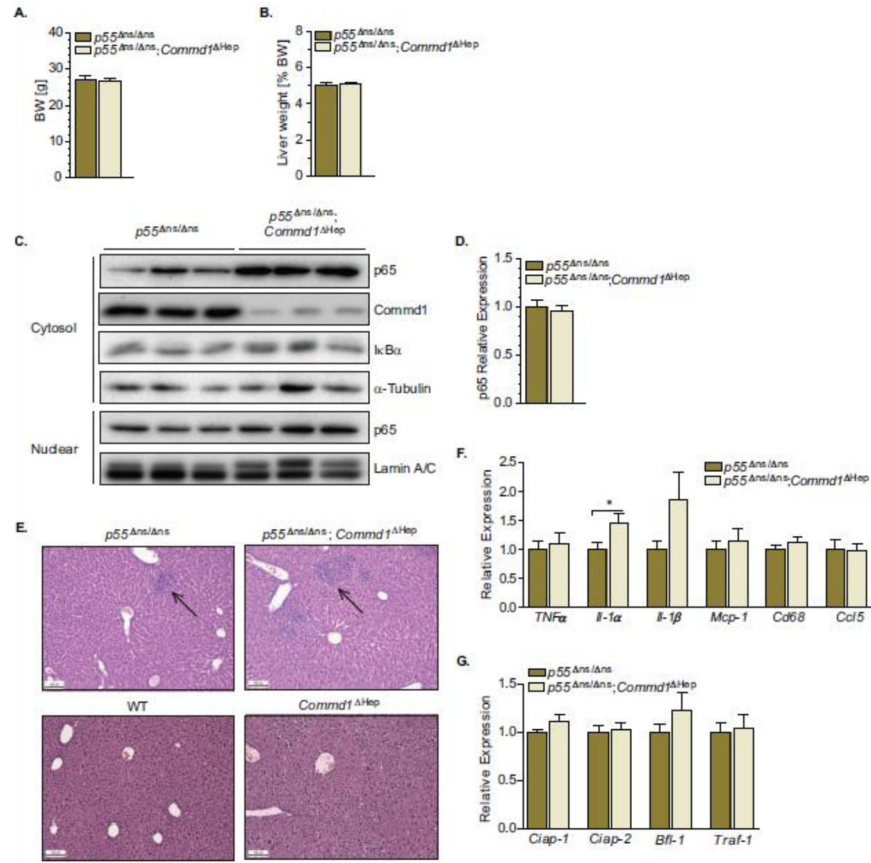


Figure 4. Loss of Commd1 function in hepatocytes does not increase Tnf-induced NF-κB activity (A) Body weight (BW) and (B) liver weight (represented as %BW) of chow-fed $p55^{ns/ns}$ and $p55^{ns/ns}; Commd1^{Hep}$ mice. (C) Cytosolic levels of hepatocyte p65 and IκBα/NFκBia) and nuclear levels of hepatocyte p65 of three representative mice per group of $p55^{ns/ns}$ and $p55^{ns/ns}; Commd1^{Hep}$ mice determined by immunoblot analysis. (D) Relative gene expression of $p65$ in liver of $p55^{ns/ns}$ and $p55^{ns/ns}; Commd1^{Hep}$ mice, determined by qRT-PCR. (E) H&E staining of hepatic tissue from chow fed mice after 4-hour fasting. Representative images per group are shown. Inflammatory foci are marked with arrows. Scale bars represent 100 μm. (F and G) Relative mRNA expression of (F) proinflammatory cytokines, macrophages markers, and (G) anti-apoptotic genes, as determined by qRT-PCR. All values per group are shown as mean ± SEM. Statistical significance was determined versus control $p55^{ns/ns}$ group. * $P < 0.05$

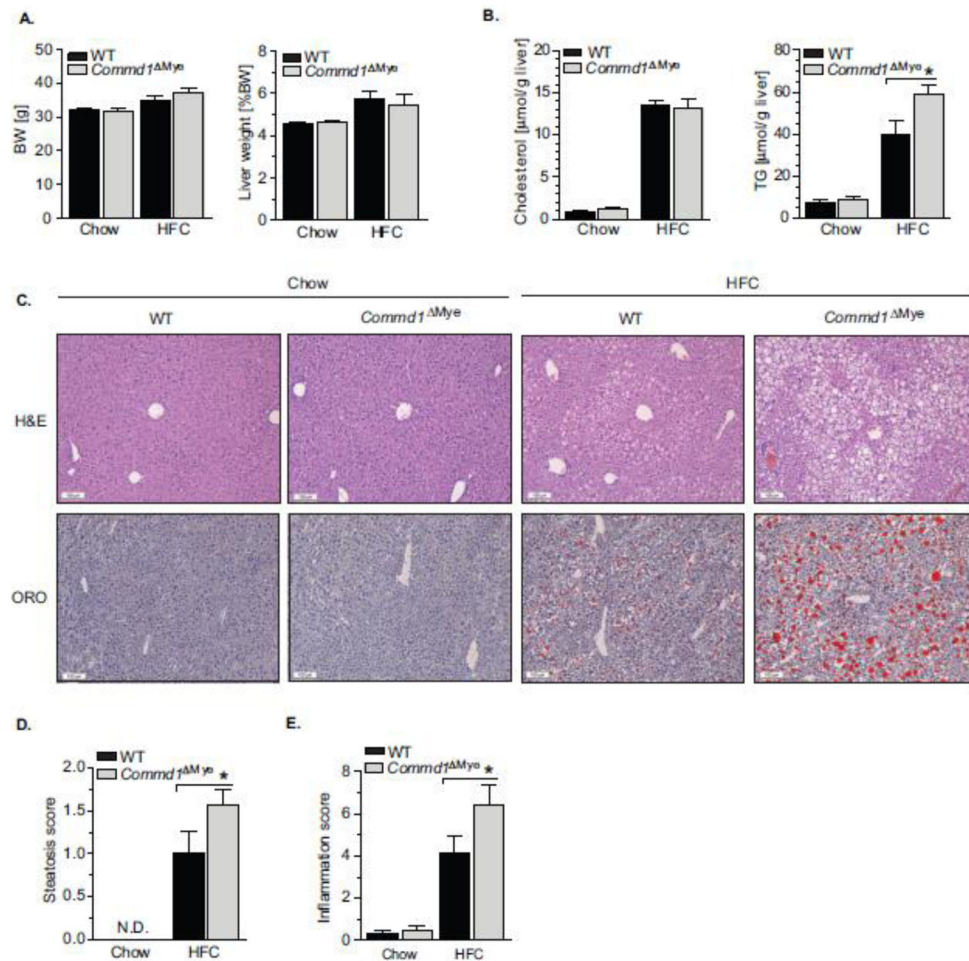


Figure 5. Myeloid *Commd1* deficiency exacerbates HFC-induced lipid accumulation
 (A) Body weight (BW) and liver weight, represented as % of BW, of WT and *Commd1*^{Hep} mice after 12 weeks on HFC diet and of control chow-fed groups. (B) Hepatic total cholesterol and triglyceride levels. (C) H&E and ORO staining of hepatic tissue from chow- and HFC-fed mice after 4-hour fasting. Representative images per group are shown. Scale bars represent 100 μm . (D) Histological evaluation of liver steatosis. Steatosis was not present in chow-fed mice (N.D. = not detected). (E) Histological evaluation of inflammation: inflammation score was based on the number of inflammatory foci per five random fields at 200x. All values per group are shown as mean \pm SEM. Statistical significance was determined versus WT control group on each diet. * $P < 0.05$.

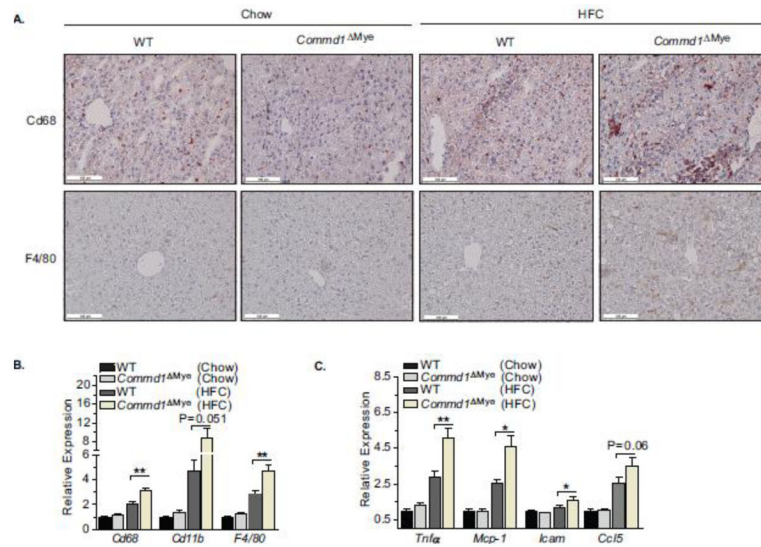


Figure 6. HFC diet-induced liver inflammation is increased upon depletion of myeloid Commd1
 (A) Immunohistochemistry staining of the macrophage markers Cd68 and F4/80. Representative images per group are shown. Scale bars represent 100 μ m. (B and C) Relative mRNA expression of (B) macrophage and monocyte markers, and (C) proinflammatory cytokines. All values per group are shown as mean \pm SEM. Statistical significance was determined versus WT control group on each diet. * $P < 0.05$, ** $P < 0.01$.