Identification of a novel multiprotein complex in cargo sorting that preserves metabolic pathways in the liver
Fedoseienko, Alina

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CHAPTER 6

COMMD9 controls plasma cholesterol homeostasis but not inflammation.

Alina Fedoseienko, Melinde Wijers, Niels Kloosterhuis, Nicolette Huijkman, Daphne Dekker, Henk van der Molen, Jan Albert Kuivenhoven, Marten Hofker, Bart van de Sluis

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Alina Fedoseienko, Melinde Wijers, Niels Kloosterhuis, Nicolette Huijkman, Daphne Dekker, Henk van der Molen, Jan Albert Kuivenhoven, Marten Hofker, Bart van de Sluis

Abstract
The Copper Metabolism MURR1 Domain (COMMD) family of proteins consists of ten members and is characterized by the carboxy-terminal COMM domain. Via this domain the COMMD proteins can interact with each other, but except for COMMD1 the cellular functions of the other COMMD proteins remain unclear. COMMD1 negatively regulates inflammation by inhibiting NF-κB signaling in a cell-type specific manner, as myeloid but not hepatic COMMD1 deficiency exacerbates liver inflammation. However, COMMD1 in hepatocytes preserves plasma cholesterol homeostasis by facilitating the endosomal trafficking of the low-density lipoprotein receptor (LDLR). COMMD1 can, together with other COMMD proteins, form stable multiprotein complexes; this indicates that COMMD proteins probably act in concert, but it is still unknown which COMMD proteins act with COMMD1, and in which cellular processes. In this study we assessed the contribution of myeloid COMMD9 in liver inflammation, and that of hepatic COMMD9 in clearing circulating cholesterol in mice. Like mice and dogs deficient in COMMD1, hepatic Commd9 knockout mice are hypercholesterolemic. On the other hand, myeloid COMMD9 depletion does not aggravate diet-induced liver inflammation, as we found after COMMD1 inactivation. Interestingly, although Commd9 ablation destabilizes COMMD1 in hepatocytes, deletion of Commd9 in macrophages does not affect the protein levels of COMMD1. These data suggest that COMMD1 and COMMD9 together control cholesterol homeostasis in hepatocytes, but do not act together in myeloid cells to inhibit inflammation; this indicates
that the compositions of the multi-COMMD protein complexes are cell type-specific to regulate distinct cellular processes.

**Introduction**

Copper Metabolism MURR1 Domain (COMMD) proteins are small ubiquitously expressed molecules characterized by a unique carboxyl-terminal homology domain. Together these proteins form the COMMD family, which consists of a total of ten members. Very little is known about the cellular functions of the COMMD proteins, except for COMMD1, the first identified member of this family. COMMD1 was initially discovered as an import factor in biliary copper excretion, as COMMD1 deficient Bedlington terriers progressively accumulate copper in their livers. The role of COMMD1 in hepatic copper homeostasis was corroborated in liver specific knockout mice, and further by its interaction with ATP7B, a copper transporting transmembrane protein which excretes copper into the bile canaliculus. Recently we found that COMMD1 is a critical component of the CCC (COMMD-CCDC22-CCDC93) complex, and that loss of COMMD1 results in destabilization of this complex. The CCC complex, in concert with retromer and the WASH (Wiskott–Aldrich syndrome protein and SCAR homologue) complex, facilitates the endosomal trafficking of the ATP7A, also a copper transporting protein homologues to ATP7B. Moreover, in addition to their role in ATP7A/7B trafficking, the CCC and WASH complexes were recently linked to the endosomal trafficking of the low-density lipoprotein receptor (LDLR) to preserve homeostatic levels of circulating LDL cholesterol. Along with its role in cholesterol and copper homeostasis, COMMD1 expression in myeloid lineage is also essential for the control of inflammation, as demonstrated by different inflammatory disease models, including non-alcoholic fatty liver disease (NASH).
Remarkably, all COMMD members are able to interact with each other\(^1\) and, through the COMM domain, have the ability to bind to the CCC-complex\(^10\). However, it is still unclear whether the COMMD proteins act together to regulate particular cellular processes. In this study we found that myeloid COMMD1 deficiency causes a reduction in a subset of COMMD proteins, including COMMD9. To assess the role of myeloid COMMD9 in inflammation we depleted Commd9 in myeloid cells and induced liver inflammation by feeding our mouse model a high-fat high cholesterol diet. Removal of COMMD9 in myeloid cells did not affect the protein levels of COMMD1, nor exacerbate diet-induced liver inflammation in mice. To the contrary, hepatic depletion of Commd9 was found to destabilize COMMD1, as well as to elevate plasma cholesterol levels; this suggests that COMMD1 and COMMD9 act together in hepatocytes but not in myeloid cells to control cholesterol homeostasis and inflammation, respectively.

**Materials and methods**

**Animals**

Mice with the conditional Commd9 allele (Commd9\(^{F/F}\)), which contains LoxP sites flanking exon 3, were generated by means of blastocyst injection, using an embryonic stem cell line (EPD0136_6_D10) obtained from The Knockout Mouse Project. Animals were genotyped by PCR using the following primers: 5′-AAG GTGGA AACAC ATAGC CAG-3′\(_1\)_ and 5′-TTA CTAGG CAACC CTGCA TTG-3′. To obtain myeloid specific Commd9 mice, mice with the conditional Commd9 allele were crossed with LysM-Cre transgenic mice\(^11\). All mice (n=7-10) were individually housed males, fed ad libitum with either a standard rodent chow diet (RMH-B, AB Diets, the Netherlands) or, starting at 10 weeks of age, a high-fat, high-cholesterol (HFC) diet (45% calories from butter fat, and 0.2% cholesterol, SAFE Diets).
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HFC feeding lasted for 12 weeks. COMMD1^{+/+} and COMMD9^{+/+} mice were injected retro-orbitally with Ad-Cre or Ad-LacZ virus particles (1 \times 10^{11} particles), and were sacrificed following a 4-hour morning fasting period. Tissues for mRNA and protein expression analysis were snap-frozen in liquid nitrogen and stored at -80^\circ C until further analysis. Blood was drawn by means of heart puncture, and plasma was isolated by centrifugation at 3000 rpm for 10 min at 4^\circ C. All animal studies were approved by the Institutional Animal Care and Use Committee, University of Groningen (Groningen, the Netherlands).

**Isolation and culture of bone marrow macrophages**

Bone marrow cells isolated from either WT or Commd9^{ΔMye} mice were cultured and differentiated into macrophages, as previously described 12.

**Cholesterol and triglyceride analysis in plasma and liver homogenates**

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

**Gene expression analysis**

Bone marrow derived macrophages (BMDMs) were grown in triplicate and lysed with QIAzol Lysis Reagent (Qiagen) upon stimulation. Pieces of murine liver of approximately 100 mg were homogenized in 1 ml QIAzol Lysis Reagent (Qiagen). Total RNA was isolated by chloroform extraction, and isopropanol-precipitated and ethanol-washed RNA pellets were dissolved in RNase/DNase free water. One microgram of RNA was used to prepare cDNA with the Transcriptor Universal cDNA Master (Roche), according to the
manufacturer’s protocol. 20 ng cDNA was used for subsequent quantitative real-time PCR (qRT-PCR) analysis using FastStart SYBR Green Master (Roche) and 7900HT Fast Real-Time PCR System (Applied Biosystems). The following PCR program was used: 50 °C/2 minutes, 95 °C/10 minutes, 40 cycles of 95 °C/15 seconds and 60 °C/1 minute. Expression data were analyzed using SDS 2.3 software (Applied Biosystems) and applying the ‘standard curve’ method of calculation. PPIA expression was used as an internal control. Primer sequences are listed in Table S1.

Western Blotting

For Western blotting, total cell/tissue lysates were obtained using 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 (Roche). Protein concentration was determined using Bradford assay (Biorad). Thirty micrograms of protein were separated using SDS-PAGE and transferred to Amersham™ Hybond™-P PVDF Transfer Membrane (GE Healthcare; RPN303F). Membranes were blocked in 5% milk in tris-buffered saline-0.01% Tween20 and incubated with the indicated antibodies. Membranes were visualized using a ChemiDoc™ XRS + System (Bio-Rad) using Image Lab software version 5.2.1 (Bio-rad).

Antibodies

In the described procedures we used the following antibodies: rabbit anti-COMMD1 (11938-1-AP, Proteintech Group), rabbit anti-COMMD2 (ab110893, Abcam), rabbit anti-COMMD3 (ab176583, Abcam), rabbit anti-COMMD4 (ab115169, Abcam), rabbit anti-COMMD5 (10393-1-AP, Proteintech Group), rabbit anti-COMMD7 (ab96091, Abcam), rabbit anti-COMMD8 (25237-1-AP, Proteintech Group), rabbit anti-COMMD9 (192-AP, custom made, Starokadomskyy, 2013), rabbit anti-COMMD10 (GTX121488, GeneTex), mouse anti-β-
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Actin (A5441, Sigma-Aldrich), rabbit anti-CCDC22 (16636-1-AP, Proteintech Group), rabbit anti-CCDC93 (20861-1-AP, Proteintech Group), HRP-conjugated goat anti-rabbit IgG (H + L) (170-6515, Bio-Rad), HRP conjugated goat anti-mouse IgG (H + L) (170-6516, Bio-Rad).

Statistical analysis

BMDMs data were the average value result of three independent experiments with triplicate per each condition ± SEM. Mouse data showed average values ±SEM. Analyses were performed using GraphPad version 6.05 (GraphPad software). The Student’s t-test was used to test the significance. For all experiments a P-value of <0.05 was considered statistically significant.

Results

Myeloid depletion of Commd1 results in reduced COMMD9 levels

We recently demonstrated that loss of myeloid COMMD1 augments the inflammatory response ⁸,⁹. In addition, COMMD1 deficiency results in decreased expression of a subset of COMMD proteins in hepatocytes (Chapter 4). To access the impact of deletion of myeloid Commd1 on the protein levels of other COMMD members we studied COMMD protein levels in bone marrow derived macrophages (BMDM) isolated from Commd1 myeloid specific knockout mice ⁹. As shown in figure 1A, the protein expression of COMMD5, COMMD9 and COMMD10 was markedly reduced, whereas the levels of COMMD3 and COMMD4 were slightly decreased after Commd1 ablation. The mRNA levels of the Commds were unaffected in COMMD1 deficient BMDM (Fig. 1B). These results are in line with our previous observations (Chapter 4), suggesting that COMMD1 is also essential for the
function of a subset of COMMD proteins.

**Figure 1.** COMMD1 depletion in macrophages results in reduced levels of COMMD9.

A. Protein levels of COMMD family member proteins in bone-marrow derived macrophages isolated from wild-type (WT) and myeloid specific Commd1 knockout mice (Commd1\(^{ΔMye}\)) determined by Western blot analysis (n=3). B. mRNA levels of COMMD family members in bone marrow macrophages from WT and Commd1\(^{ΔMye}\) mice (n=9), as determined by quantitative RT-PCR. All values per group shown as mean ± SEM.

*Myeloid COMMD9 deficiency does not affect LPS-induced inflammation*

As depletion of Commd1 in macrophages resulted in a remarkable reduction of COMMD9 levels, we decided to determine *in vivo* the role of myeloid COMMD9 in inflammation. To inactivate COMMD9 in the myeloid lineage (Commd9\(^{ΔMye}\)) we crossed mice harboring loxP sites flanking exon 3 of the Commd9 gene (Fig. 2A) with LysM-Cre transgenic mice. BMDM from control mice and Commd9\(^{ΔMye}\) were cultured and the expression of COMMD
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proteins was assessed by immunoblot analysis. Although \textit{Commd9}\textsuperscript{ΔMye} BMDM showed the expected loss of COMMD9 expression, we observed no clear differences in the levels of other COMMD proteins (Fig. 2B). Subsequently, we determined whether depletion of COMMD9 affects the inflammatory response in macrophages.

We studied the expression of a number of inflammatory and NF-κB target genes in BMDM upon lipopolysaccharide (LPS) stimulation (0, 3, and 24 hours). Between control and \textit{Commd9}\textsuperscript{ΔMye} BMDM we observed no significant differences in the expression of the studied genes, with the exception of the anti-inflammatory cytokine \textit{Il-10} (Fig. 2C). At 24 hours, the \textit{Il-10} mRNA levels were slightly increased in \textit{Commd9}\textsuperscript{ΔMye} BMDM compared to control cells.

\textit{Ablation of myeloid Commd9 does not aggravate diet-induced liver inflammation}

Previously we reported that myeloid COMMD1 suppresses inflammation in different inflammatory disease models, including non-alcoholic fatty disease (NASH)\textsuperscript{8,9}. As shown above, myeloid depletion of \textit{Commd1} resulted in reduced COMMD9 levels. We therefore assessed the role of myeloid Commd9 in liver inflammation during the development of steatohepatitis. We fed wild-type (WT) (n = 6-10) and \textit{Commd9}\textsuperscript{ΔMye} mice (n = 7-8) either chow or HFC diet for 12 weeks. \textit{Commd9} ablation in myeloid cells did not lead to differences in body and liver weight (Fig.3 A, B). Plasma and liver triglyceride and cholesterol levels were also not affected by the loss of myeloid COMMD9 upon HFC diet treatment (Fig. 3C, D, E, F). HFC diet induced steatosis in both groups was confirmed by H&E staining of liver sections (Fig. 3 G)
Figure 2. COMMD9 deficient macrophages do not show impaired NF-κB-mediated gene expression.

A. Schematic representation of the Commd9 gene-targeting strategy to generate myeloid-specific Commd9 knockout mouse, including map of Commd9 exons and targeting vector with loxP sites (black triangles), FRT
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sites (black rhombs) and neomycin selection gene (Neo). B. Protein levels of COMMD family member proteins in lysates of bone-marrow derived macrophages isolated from wild-type (WT) and myeloid specific Comm9 knockout mice (Comm9ΔMye), determined by Western blot analysis (n=3). C. Relative mRNA expression of inflammatory cytokines and NF-κB target genes in bone marrow macrophages from WT (white bars) and Comm9ΔMye mice (grey bars) stimulated with LPS for 0, 3 or 24 hours. All values per group shown as mean ± SEM, * p<0.05. PPIA expression used as an internal control.

Next, the level of liver inflammation was assessed by determining the gene expression of proinflammatory cytokines such as Tnf-α, Il-1α, Il-1β1, Mcp-1 and Ccl5 in livers of chow and HFC-diet fed WT and Comm9ΔMye mice, but no differences between the two genotypes were found. In addition, the gene expression of F4/80 (marker of mature macrophages, highly expressed by Kupffer cells), and Cd11b (a migratory marker of blood-derived monocytes), was also unaffected by myeloid COMMD9 deficiency (Fig. 3G). In conclusion, depletion of Comm9 in myeloid cells does not exacerbate diet-induced liver inflammation, and does not affect the progression of steatosis upon 12 weeks of HFC diet feeding.

Hepatic Comm9 depletion results in elevated plasma cholesterol levels

We recently discovered that hepatic COMMD1 and COMMD6 control plasma cholesterol levels (7, Chapter 4), and since all COMMD proteins can associate with the CCC-complex 7,10 we were prompted to elucidate the role of hepatic COMMD9 in cholesterol homeostasis. We ablated Comm9 specifically in hepatocytes by intravenous injection of recombinant adenovirus carrying Cre recombinase into Comm9F/F. Yet, to confirm that this approach is suitable for studying the COMMD family in cholesterol homeostasis, we first intravenously injected conditional Comm1 knockout mice (Comm1F/F) with Cre adenovirus (Ad-Cre) to inactivate COMMD1. As a negative control we injected Comm1F/F mice with LacZ adenovirus (Ad-LacZ). We sacrificed the mice 3 weeks after viral injection, and determined
Figure 3. Myeloid depletion of murine *Commd9* does not affect diet-induced liver inflammation.

A. Body weight (BW), and D. liver weight (% of body weight) of myeloid specific *Commd9* knockout mice (*Commd9ΔMye*) and WT mice (n=7-10) fed chow or a high-fat high-cholesterol (0.1%) (HFC) diet for 12 weeks.

B. Plasma triglyceride (TG), and C. total cholesterol levels of myeloid specific *Commd9* knockout mice (*Commd9ΔMye*) and WT mice (n=7-10) fed a high-fat high-cholesterol (0.1%) (HFC) diet for 12 weeks. E.
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Hepatic total cholesterol and F. triglyceride levels. Liver lipids extracted from snap-frozen mouse livers using Bligh-Dyer method for lipid extraction and analyzed with colorimetric assay. G. H&E staining of hepatic tissue from 4-hour fasted chow- and HFC-fed mice. H&E staining performed on paraffin-embedded samples. Representative images shown per group. Scale bars represent 100 µm. H. Relative liver mRNA expression of proinflammatory cytokines and NF-κB target genes, Il-1α, Il-1β, Tnfα, Mep-1, Ccl5 and of macrophage and monocyte markers Cd11b and F4/80. All values per group shown as mean ± SEM.

the hepatic COMMD1 levels and plasma cholesterol concentrations. The mRNA and protein levels of COMMD1 were markedly reduced after Ad-Cre injection (Fig. 4A, B).

Inactivation of COMMD1 resulted in a significant increase in plasma cholesterol levels, as we also previously found in liver specific Commd1 knockout mice, in which Commd1 was deleted by transgenic expression of Cre recombinase 7,9. After validating this approach, to study the function of hepatocyte COMMD proteins in cholesterol homeostasis, we deleted hepatic Commd9. Intravenous Ad-Cre injection in Commd9F/F resulted in an 80% reduction of Commd9 mRNA expression (Fig. 4D), and a marked decrease in COMMD9 levels (Fig. 4E). Interestingly, hepatic COMMD9 deficiency is accompanied by an almost complete loss of COMMD1 and its interacting partner CCDC22, but to a lesser extent CCDC93, whereas the expression of COMMD3 was not affected by Commd9 depletion (Fig. 4E). Like hepatic Commd1 deletion, disruption of Commd9 in hepatocytes also caused a significant increase in circulating total cholesterol (Fig. 4C, F). Taken together, these data demonstrate that ablation of hepatic Commd9 impairs the integrity of the CCC-complex (COMMD1, CCDC22, CCDC93), accompanied by elevated plasma cholesterol levels.
Figure 4. Hepatocyte Commd9 depletion results in elevated cholesterol levels.

A. Relative liver mRNA expression of Commd1 in Commd1 Floxed/Floxed (Commd1 F/F) mice infected with control adenovirus (Ad-LacZ) or adenovirus carrying Cre recombinase (Ad-Cre) (n=4).

B. Protein levels of COMMD1 in livers of Commd1 F/F mice infected with control adenovirus (Ad-LacZ) or adenovirus carrying Cre recombinase (Ad-Cre), determined by Western blot analysis (n=4). β-actin used as loading control.

C. Plasma cholesterol levels in Commd1 F/F mice infected with control adenovirus (Ad-LacZ) or adenovirus carrying Cre recombinase (Ad-Cre) (n=4).

E. Relative liver mRNA expression of Commd9 in Commd9 F/F mice infected with control adenovirus (Ad-LacZ) or adenovirus carrying Cre recombinase (Ad-Cre) (n=4).

F. Cholesterol levels before viral injection and after adenovirus injection (AV-LacZ Av-Cre) (n=4).
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Floxed/Floxed (Commd1 F/F) mice infected with control adenovirus (Ad-LacZ) or adenovirus carrying Cre recombinase (Ad-Cre) (n=4). B. Protein levels of indicated proteins in livers of Commd9 F/F mice infected with Ad-LacZ or Ad-Cre, determined by Western blot analysis (n=4). β-actin used as loading control. C. Plasma cholesterol levels in Commd9 F/F mice infected with Ad-LacZ or Ad-Cre (n=4).

Discussion

Prior results suggested the existence of numerous multiprotein complexes composed of different COMMD proteins (10,13, Chapter 4). To better understand the compositions and functions of the COMMD-associated protein complexes, we studied the role of COMMD9 in inflammation and cholesterol homeostasis in murine myeloid and liver cells, respectively. The present study demonstrates that in contrast to COMMD1, myeloid COMMD9 does not control inflammation, as COMMD9 deficiency in BMDM does not affect the inflammatory response after LPS stimulation. Furthermore, diet-induced liver inflammation is also not exacerbated by the loss of myeloid COMMD9. However, COMMD9 in hepatocytes controls cholesterol homeostasis, as does COMMD1 7.

Although both COMMD1 and COMMD9 can interact with each other 10,13, and inhibit NF-κB-mediated inflammation in vitro1, we were not able here to provide evidence that COMMD9 suppresses inflammation in vivo, as we had demonstrated for COMMD1 8,9. In contrast to COMMD1, COMMD9 is not required for the protein stability of other COMMD proteins in BMDM; this suggests that COMMD9 participates in a protein complex distinct from COMMD1 that regulates NF-κB signaling 10,14.

Interestingly, in contrast to myeloid COMMD9, hepatic COMMD9 inactivation resulted in reduction of COMMD1, as well as other components (CCDC22 and CCDC93) of the CCC complex. As expected, this inactivation of the CCC complex resulted in elevated plasma cholesterol levels. We previously reported that patients with mutations in CCDC2215,
and mice or dogs deficient in COMMD1, are hypercholesterolemic. In mice and dogs, loss of COMMD1 impairs the integrity of the CC complex, resulting in mislocalization of LDLR. Remarkably, although both COMMD3 and COMMD9 levels are reduced in hepatocytes lacking COMMD1 (Chapter 4) the levels of COMMD3 were not affected by Commd9 ablation. The explanation for this discrepancy is unclear but it is possible that the level of COMMD1 reduction in COMMD9 deficient hepatocytes does not mimic the complete loss of COMMD1 in Commd1 knockout cells, which is necessary to disturb the levels of COMMD3. Since the level of plasma cholesterol increase by Commd1 ablation is higher than by Commd9 deletion (Fig. 4C, F; 50% versus 30% increase) it is interesting to speculate that COMMD3 also participates in the CCC complex to facilitate the endosomal trafficking of LDLR, and that COMMD3, which is still present in COMMD9 deficient hepatocytes, can partially take over the loss of COMMD1 and COMMD9.

Taken together, this study identified COMMD9 as a crucial member of the COMMD family, participating in the CCC complex to control cholesterol homeostasis but not inflammation. These data support the hypothesis that COMMD proteins can form multiprotein complexes, comprising different combinations of COMMD proteins in a tissue-specific manner to regulate particular cellular processes. Further research is needed to unravel the compositions and functions of these COMMD-associated protein complexes.
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References


