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IL23 signaling is not an important driver of liver inflammation and fibrosis in murine non-alcoholic steatohepatitis models

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Abstract:	Non-alcoholic fatty liver disease (NAFLD), represents an unmet medical need that can progress to non-alcoholic steatohepatitis (NASH), which, without intervention, can result in the development of cirrhosis and hepatocellular carcinoma (HCC). Inflammation is a pathological hallmark of NASH, and targeting key inflammatory mediators of NASH may lead to potential therapeutics for the disease. Herein, we aimed to investigate the role of IL-23 signaling in the disease progression in murine NASH models. We showed that recombinant IL-23 can promote IL-17 producing cell expansion in the liver and that these cells are predominately T cells and Mucosal Associated Invariant T cells (MAITs). Reciprocally, we found that IL23R is necessary for the expansion of T cells and MAIT cells in the western diet (WD) diet induced NASH model. However, we did not observe any dramatic differences in liver inflammation and fibrosis between wild type and Il23r-/- mice in the same NASH model. Furthermore, we found that Il23r deletion does not impact liver inflammation and fibrosis in the choline-deficient, L-amino acid-defined and high-fat diet (CDA-HFD) induced NASH model. Based on these findings, we therefore propose that IL-23 signaling is not a crucial determinant of NASH pathogenesis in preclinical models and targeting this pathway may not be sufficient to ameliorate the disease progression in NASH patients.
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1 **IL23 signaling is not an important driver of liver inflammation and fibrosis in murine non-**
2 **alcoholic steatohepatitis models**
3
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16 **Abstract**
17 Non-alcoholic fatty liver disease (NAFLD), represents an unmet medical need that can progress
18 to non-alcoholic steatohepatitis (NASH), which, without intervention, can result in the
19 development of cirrhosis and hepatocellular carcinoma (HCC). Inflammation is a pathological
20 hallmark of NASH, and targeting key inflammatory mediators of NASH may lead to potential
21 therapeutics for the disease. Herein, we aimed to investigate the role of IL-23 signaling in the
22 disease progression in murine NASH models. We showed that recombinant IL-23 can promote IL-

23 17 producing cell expansion in the liver and that these cells are predominately $\gamma\delta$ T cells and
24 Mucosal Associated Invariant T cells (MAITs). Reciprocally, we found that IL23R is necessary for
25 the expansion of $\gamma\delta$ T cells and MAIT cells in the western diet (WD) diet induced NASH model.
26 However, we did not observe any dramatic differences in liver inflammation and fibrosis between
27 wild type and Il23r-/- mice in the same NASH model. Furthermore, we found that Il23r deletion
28 does not impact liver inflammation and fibrosis in the choline-deficient, L-amino acid-defined and
29 high-fat diet (CDA-HFD) induced NASH model. Based on these findings, we therefore propose
30 that IL-23 signaling is not a crucial determinant of NASH pathogenesis in preclinical models and
31 targeting this pathway may not be sufficient to ameliorate the disease progression in NASH
32 patients.
33

34 **Introduction**

35 Non-alcoholic fatty liver disease (NAFLD) is defined as a chronic liver disease that imparts excess
36 lipid accumulation in the liver in the absence of secondary causes such as viral infections or
37 alcohol abuse[1, 2]. NALFD can progress from simple hepatic steatosis to non-alcoholic
38 steatohepatitis (NASH) characterized by inflammation and fibrosis, which is a significant risk
39 factor for cirrhosis and hepatocellular carcinoma (HCC) [3-6]. Within the past decades, the
40 epidemic of obesity has led to the sharp rise of NALFD/NASH occurrence [2, 7]. However, there
41 are no FDA-approved therapies for NASH driven chronic liver disease, which may be largely due
42 to our limited understanding of molecular underpinnings of liver inflammation and fibrosis.
43

IL-23 is a cytokine that has been implicated in IL-17 driven pathologies such psoriasis, colitis, and autoimmune diseases [8-11]. As an IL-12 cytokine family member, IL-23 is composed of a heterodimer of IL-12p40 subunit and IL-23p19 subunit (IL-23p19/p40) which signals through the IL-23R and IL-12Rβ1 dimeric receptor [12]. Mice that lack IL-23p19 demonstrate an inability to drive the expansion of pathogenic IL-17 producing cells [13-15]. All IL-17 expressing cells also express RAR-related orphan receptor gamma t (RORγt), the master transcription factor driving the TH17 signature [16, 17]. In this regard, previous studies have implicated that IL-17 producing cells promote hepatic inflammation and fibrosis[18]. It has also been reported that there is accumulation of IL-17 producing cells in the livers from NASH patients or diet induced NASH preclinical murine models [19-22]. Additionally, IL-17 has been shown to be elevated by liver damaging agents such as Carbon Tetra-Chloride and Concanavalin A in models of acute hepatitis [18, 20-23]. While these studies suggest that IL-17 producing cells may contribute to chronic liver disease, the role of IL-23 signaling in NASH has not been fully dissected.

In this study, we hypothesized that IL-23 signaling may play an important role in the NASH pathogenesis. We showed that systemic administration of recombinant IL-23 protein induces IL-17 producing cell expansion in the liver and that these cells are predominately $\gamma\delta$ T cells and Mucosal Associated Invariant T cells (MAITs). Reciprocally, genetic ablation of Il23r attenuated $\gamma\delta$ T and MAIT cell expansion in western diet (WD)-induced NASH model. However, we did not observe that Il23r deletion exhibits dramatic effects on liver inflammation, fibrosis and liver function in the same model. Similarly, we found that Il23r-/- mice are not protected from liver inflammation and fibrosis in another model, the choline-deficient, L-amino acid-defined and

66 high-fat diet (CDA-HFD) induced NASH model. Thus, these results do not support a causal role of
67 IL-23 signaling in the NASH pathogenesis and suggest that targeting IL-23 signaling may not be a
68 viable therapeutic strategy to treat NASH patients.
69
70 **Results**
71 **Recombinant IL-23 increases RORγt cell accumulation in liver**
72 In order to determine whether IL-23 is sufficient to induce hepatic expansion of RORγt positive
73 IL-17 producing cells, we intraperitoneally injected 0.5ug recombinant mouse IL-23 (rmIL-23)
74 daily for three consecutive days into mice fed on normal diet (ND) and analyzed the livers 24hrs
75 after the last injection. Administration of rmIL-23 led to a five-fold expansion of hepatic Ki67$^+$
76 RORγt cells (Figures 1A and 1B) and a two-fold increase in the percentage of Ki67$^+$ RORγt cells
77 when compared to vehicle control (Figure 1C). RORγt cells that proliferated actively were
78 identified as MAIT and γδ T Cells (Figures 1D and 1E). rmIL-23 treatment also induced an increase
79 in the percentage of hepatic neutrophils and inflammatory monocytes when compared to vehicle
80 control (Figures 1F and 1G). These results thus suggest that IL-23 is sufficient to induce RORγt cell
81 accumulation and pro-inflammatory response within the livers.
82
83 **Western Diet induced hepatic expansion of RORγt cells is dependent on IL-23R**
84 Next, we investigated whether IL-23 signaling is required for of RORγt cell accumulation in the
85 animal model of NASH. Western Diet (WD) consisting of high fat, high fructose, and added
86 cholesterol have been established to induce several NASH phenotypes including hepatic
87 inflammation, fibrosis and an increase in liver damage measured by the serum biomarkers such

88 as alanine aminotransferase (ALT), aspartate aminotransferase (AST), all in a nutritional setting
89 without liver damaging chemicals [24, 25]. We fed WT and Il23r-/- mice with normal diet (ND) or
90 WD for 20 weeks (Figure 2A) [26]. The WD induced the expansion of RORγt$^+$ $\gamma\delta$ T cells and MAIT
91 cells in livers from WT mice (Figure 2B and 2C). In Il23r-/- mice, we found that WD-induced
92 expansion of $\gamma\delta$ T cells and MAIT cells were dramatically normalized to the baseline.
93 Furthermore, by intracellular staining of liver $\gamma\delta$ T cells, we observed a significant decrease of IL-
94 17A production from Il23r-/- compared to WT. These data suggest that IL-23 plays an important
95 role in regulating IL17 producing cells in WD induced NASH model.
96

97 **IL-23 signaling is not critical for hepatic inflammation and fibrosis induced by WD in mice**
98 Having established its critical role in WD-indued RORγt cell accumulation, we explored the
99 contribution of IL-23 signaling to WD-induced liver inflammation and fibrosis. While there is a
100 clear increase of hepatic inflammation induced by WD, we did not observe any noticeable
101 differences in liver inflammation between WT and Il23r-/- livers from WD fed mice as assessed
102 by histology and pro-inflammatory gene expression (Figures 3A and 3B). On the other hand, while
103 we observed a modest, but statistically significant, reduction of pro-inflammatory monocytes in
104 the WD indued Il23r-/- liver (Figure 3C), there was no significant difference of neutrophil
105 infiltration to the liver between Il23-/- and WT mice (Figure 3D), the main myeloid cell known to
106 be recruited by IL-17 induced chemokines. In addition, we found that the serum levels of
107 Keratinocytes-derived chemokine (KC) (Figure 3E) and Interferon gamma-induced protein 10 (IP-
108 10) (Figure 3F) were not changed in Il23r-/- mice. Next, to address the role of IL23R signaling in
109 liver fibrosis in the WD-induced NASH model, we evaluated hepatic collagen content by

110 trichrome staining analysis and hepatic collagen gene expression. We did not observe a
111 significant difference of collagen content at the histology level as well as at the transcriptional
112 level (Figures 4A and 4B). Overall, these results do not suggest IL-23 signaling as the main driver
113 of liver inflammation and fibrosis in the WD-induced NASH model.
114
115 **IL-23 signaling does not contribute to WD induced liver dysfunction**
116 Next, we examined the impact of Il23r deletion on liver function. In this regard, we measured
117 several serum biomarkers of liver function. The results showed that there is little difference of
118 serum ALT/AST, alkaline phosphatase (AP), albumin, cholesterol, and triglyceride levels between
119 WT and Il23r-/- mice fed on WD (Figure 5C-5H). Similarly, IL-23R deficiency appears not to affect
120 the WD-induced whole-body weight as well as liver weight gains (Figure 5A and 5B). Collectively,
121 these data suggests that IL-23 signaling may not contribute to liver dysfunction caused by WD-
122 induced metabolic imbalance.
123
124 **IL-23 signaling does not contribute to liver inflammation and fibrosis in the CDA-HFD model of**
125 **NASH**
126 To complement our findings in WD-induced NASH model, we sought to determine whether IL23
127 signaling contributes to the pathogenies of NASH in another animal model. In this regard, we
128 chose the CDA-HFD model (Figure 6A) because this model has been demonstrated to recapitulate
129 steatosis, inflammation, and advanced fibrosis in liver [27]. The CDA-HFD significantly induced
130 the expansion of RORγ+ $\gamma\delta$ T Cells and MAIT cells in mouse livers (Figures 6B and 6C). Consistent
131 to our observation in WD-induced NASH model, we found no impact of IL-23R depletion on liver

132 inflammation and fibrosis by histology (Figures 6D). Furthermore, no significant changes in
133 hepatic inflammatory or fibrotic gene expression were detected between WT and Il23r-/- mice
134 fed on CDA-HFD (Figure 6G). In addition, we did not observe any significant change in body
135 weight, liver weight, and a variety of serum biomarkers for liver function between WT and Il23r-
136 /- mice. Taken together, these results suggest that IL-23 signaling does not contribute to hepatic
137 inflammation and fibrosis in the CDA-HFD NASH model.
138

139 **Discussion**

140 NAFLD/NASH is an unmet medical need that is increasingly common around the world. The
141 incidence of NAFLD world-wide is approximately 25%, and the global prevalence of NASH patients
142 from NAFLD biopsied patients was estimated to be 59.1% based on Meta-analysis data [7, 28].
143 There are currently no approved therapies for NAFLD/NASH, and pro-inflammatory pathways
144 have been proposed to be a class of appealing targets for this complex disease [29]. In this regard,
145 it came to our attention that hepatic IL-17 producing cells have been shown to promote liver
146 inflammation and dysfunction [18-21]. However, genetic dissection of this pathway, particularly
147 its upstream regulator IL-23, in the preclinical NASH models is lacking and the target candidacy
148 of this Il-17/Il-23 axis in NASH is yet to be fully established.
149

150 In this context, we therefore chose to investigate the contribution of Il-23 signaling to NASH
151 pathogenesis by testing IL-23R deficient mice in animal models of NASH. Our data showed that,
152 while recombinant IL-23 is sufficient to drive IL-17A producing cell expansion and pro-
153 inflammatory myeloid cell infiltration in liver, Il-23r-/- mice are not protected from liver

inflammation and fibrosis in two NASH models, suggesting the contribution of Il-23 signaling to NASH pathogenesis is minimal. These observations thus challenge the assumption that IL-17 producing cells that have been shown to be present in NASH patient liver samples may play a causal role in the disease pathogenesis[30]. It should also be noted that we cannot rule out IL-23's contribution to non-NASH liver fibrosis as some reports suggest IL-23 signaling plays a role in cholestatic or viral driven liver fibrosis [18, 31]. Nevertheless, the dispensability of IL-23 signaling in NASH driven liver inflammation is intriguing given its critical role in a wide variety of pro-inflammatory diseases. Since it is well documented that many inflammatory factors such as cytokines and PAMPs are elevated in NASH models, it is not inconceivable that the accumulation of these factors may mask any effects of IL-23 in the NASH models [29, 32]. Further studies are warranted to dissect the potential crosstalk between IL-23 and other proinflammatory cytokines during the pathogenesis of NASH.

In summary, we present the evidence that IL-23r -/- mice are not protected from liver inflammation and fibrosis in two NASH preclinical models, thus suggesting that IL-23 signaling may not be an effective therapeutic target for NASH. Our study also supports the necessity of leveraging genetic models to validate drug targets when possible and the notion that the overall role of IL-23/IL-17 axis in NASH may need to be re-evaluated.

Material and Methods

Mouse studies

175 Il23r-/- mice were generated as described previously [33], the control group were littermate wild
176 type mice. Diets used in this study were purchased from Research Diets; Normal Diet (ND) was
177 compared either to Western Diet (WD) (Diet #D19021501) composed of 40% kcal Fat, 22% kcal
178 Fructose, and 1.25% Cholesterol or Choline Deficient L-Amino Acid Derived High Fat Diet (CDA-
179 HFD) (Diet #A06071302) composed of 60% kcal Fat, 0.1% Methionine, and no added choline. All
180 mice started the diets at 8 weeks of age, and all mice used were males. The ND vs WD cohort
181 were challenged with the diet for 20 weeks. The ND vs CDA-HFD cohorts were on the diets for 9
182 weeks. C57Blk/6 mice from Jackson Laboratory were used for the Intraperitoneal (IP) injects of
183 PBS vs IL-23. Recombinant murine IL-23 was purchase from R&D, mice were IP with either PBS or
184 0.5ug IL-23 for three consecutive days and livers were harvested 24hrs after last injection. All
185 animal activity were performed as required by the Institutional Animal Care and Use Committee
186 (IACUC) of Genentech Inc., Animal Welfare Act, and in Accordance with the Guide for the care
187 and use of laboratory animals (The Guide).
188
189 **Liver digestion and flow cytometry**
190 Upon CO_2 euthanasia, serum was collected, and livers were perfused with 1X PBS, via the portal
191 vein. Livers were collected for either histology, snap frozen, or tissue processing for non-
192 parenchymal cell (NPC) isolation. All liver samples per cohort were processed at the same time
193 by transferring the livers in c-tubes (Miltenyi) and adding 5mL of digestion media consisting of
194 0.2% Collagenase Type 2 (Worthington), 0.1% DNAse I (Roche), 1% BSA (Sigma), in RPMI. Samples
195 were digested using MACS Miltenyi Dissociator using liver dissociation settings, and incubating
196 samples at 37degrees C for 30mins in shaker at 120rpm. After digestion incubation, samples were

197 centrifuged at 1600rpm for 5mins, ACK treated pellets were resuspended in 1X PBS and passed
198 through a 70um cell strainer. Samples were pelleted, and resuspend in 15% Percoll, centrifuged
199 for 1600rpm for 15mins without brake. These pellets were the non-parenchymal cell (NPC)
200 fraction, free of hepatocytes. The NPCs were then resuspended in 1X PBS, stained with
201 LIVE/DEAD fixable dye (Invitrogen) at a 1:1000 dilution, incubated on ice for 15mins, washed,
202 resuspended in FACS buffer (PBS + 2.5mM EDTA + 5% BSA) with FcR block (Miltenyi), and stained
203 with the appropriate conjugated fluro-antibodies. For RORgt staining, cells were
204 processed/stained using the FOXP3 Transcription Factor staining kit (BD). For intracellular
205 staining of IL-17a, NPCs were stimulated with Leukocyte Activation Cocktail with GolgiPlug (BD)
206 for 4hours in RPMI media, then cells were washed, FcR blocked, and stained with appropriate
207 antibodies. Samples were run and analyzed on Symphony analyzer (BD).
208
209 **RNA Extraction and Quantitative PCR**
210 RNA was isolated from approximately 100mg of liver tissue using 1mL Trizol using the bead
211 homogenizer Qiagen method, followed by addition of 200uL chloroform, resuspended samples
212 were centrifuged for 10mins at 13krpm, 300uL clear top aqueous layer was transferred to new
213 tube followed by the addition of 300uL 70% Ethanol. The 600uL samples were then loaded on a
214 RNeasy Mini purification column (Qiagen) for RNA isolation. RNA quantification and purity was
215 analyzed with NanoDrop 2000 (Thermo Scientific). 1ug of RNA was used for cDNA synthesis using
216 Iscript First Strand cDNA kit (BioRad). cDNA templates were combined with Taqman probes
217 (Thermo), and Taqman Universial PCR Master Mix (Thermo), and run on QuantaStudio 6 Flex
218 (Applied Biosystems).

Histology

Paraffin embedded liver tissues were sectioned and stained for Hematoxylin and Eosin staining and for Trichrome staining. Automated image analysis was conducted on trichrome stained slides to access fibrosis and inflammation. Features counted towards inflammation include inflammatory cells (lobular inflammation), primarily macrophages with some neutrophils, and areas of hepatocyte injury/ductular reaction. The trichrome stain allowed for robust identification of both fibrosis and inflammatory features.

Serum Biomarker and Cytokine Analysis

The liver chemistry panel consists of the following assays: Alanine Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (AP), Albumin (ALB), and Triglycerides (TRIG). All assays were performed on the Beckman Coulter Au480 chemistry analyzer using the analytical principle of spectrophotometry and potentiometry. (Beckman Coulter Inc., Brea CA). Serum cytokines were measured using Luminex bead assay (Millipore platform).

Quantification and Statistical Analysis

GraphPad Prism 6 was used for statistical methods using the unpaired student t-test for panels in Figure 1 and Figure 2D. All other experiments were performed using one-way ANOVA. Statistical details provided in the figure legends.

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372

373 **Figure Legends**

374 **Figure 1. rmIL-23 administration induces RORγt proliferation in liver**

375 FACS staining was performed of hepatic non-parenchymal cells (NPC) from mice administered
376 either PBS or IL-23 by Intraperitoneal (IP) injections (A-G): Representative FACS gate, IL-23
377 induced greater frequency of RORγt^{+}Ki67^{+} T Cells gated from CD3e^{+}CD4^{-}CD8^{-} (A). Total
378 RORγt^{+}Ki67^{+} T Cells, indication of proliferative cell, and total RORγt (CD4-CD3-) T Cells quantified
379 in the liver (B-C). Percentage quantification of Ki67+ of RORγt^{+}MAITs and RORγt^{+} γδ T Cells (D-E).
380 Percentage of Neutrophils and Ly6c^{+} monocytes from CD45+ Cells (F-G). Groups: Vehicle (PBS):
381 n=6, IL-23 (3x 0.5ug): n=6. Data represents mean ± S.D. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{****}p < 0.0001$,
382 two-tailed t-test.

383

384 **Figure 2. IL-23 is required for WD-induced hepatic IL-17 producing cell expansion**

385 WT or Il23r-/- mice were fed a ND or WD for 20 weeks, followed by terminal analyses (A). FACS
386 quantification of percent RORγt^{+} from γδ T Cells and RORγt^{+} from MAIT cells (CD3e^{+}TCRb^{+}CD4^{-}
387 CD8^{-}) (B-C). Percentage of IL-17A positive cells in γδ T cells from WD fed WT and Il23r-/- liver non-
388 parenchymal cells (NPCs) stimulated with Leukocyte Activation Cocktail with GolgiPlug (BD) for 4
389 hours (D). Groups: ND WT n=5, ND Il23r-/- n=5, WD WT n=7, WD Il23r-/- n=7. Data represents
390 mean ± S.D. $^{*}p < 0.05$, $^{**}p < 0.005$, one-way ANOVA.

391

392 **Figure 3. IL-23 signaling is dispensable for WD-induced hepatic inflammation**

393 Liver sectioned H&E stained images of WT and Il23r-/- mice on ND or WD (A). Liver mRNA
394 expression of *Tnf, Cd68, Ccl2, Cxcl2,* and *Cxcl10* (B). Liver FACS analysis of percent Ly6c^{+}

395 Monocytes and Neutrophils between the groups. Luminex results for serum levels of KC (E) and
396 IP-10 (F) chemokines. Groups: ND WT n=5, ND Il23r-/- n=5, WD WT n=7, WD Il23r-/- n=7. Data
397 represents mean $\pm$ S.D. *p < 0.05, **p < 0.005, ****p < 0.00005, one-way ANOVA. C/D??????
398

399 **Figure 4. IL-23 signaling does not contribute to WD-induced hepatic fibrosis**

400 Trichrome staining images of livers (A). Liver mRNA expression of *Col1a1, Col1a2,* and *Col3a1*.
401 Groups: ND WT n=5, ND Il23r-/- n=5, WD WT n=7, WD Il23r-/- n=7.
402

403 **Figure 5. IL-23 signaling does not contribute to WD-induced liver dysfunction**

404 Body weights in gram(g) (A) and percent liver weights from body weight (B). Quantification of
405 serum liver enzymes for Alanine Aminotransferase (ALT) (C), Aspartate Aminotransferase (AST)
406 (D), and Alkaline Phosphatase (AP) (E) measured. Serum Albumin protein (F), serum cholesterol
407 (G) and serum triglycerides (H) quantified. Groups: ND WT n=5, ND Il23r-/- n=5, WD WT n=7, WD
408 Il23r-/- n=7. Data represents mean $\pm$ S.D, one-way ANOVA.
409

410 **Figure 6. IL-23 signaling does not contribute to liver inflammation and fibrosis in the CDA-HFD**
411 **NASH model.**

412 WT and Il23r-/- mice were fed CDA-HFD for 9 weeks, followed by liver analysis (A). Percent
413 quantification of hepatic RORγt in $\gamma\delta$ T Cells (B) and MAITs (C). Trichrome staining images and
414 quantification of Inflammation (E) and Trichrome Collagen content (F). Liver mRNA expression of
415 *Tnf, Cd68, Ccl2, Cxcl2, Col1a1, Col1a2,* and *Col3a1* (G). Groups: ND WT n=3, ND Il23r-/- n=3, CDA-
416 HFD WT n=6, CDA-HFD Il23r-/- n=6. Data represents mean $\pm$ S.D. ***p < 0.0005, one-way ANOVA.

417

418 **Figure 7. IL-23 signaling does not contribute to CDA-HFD-induced liver dysfunction.**

419 Body weights in gram (g) (A) and percent liver weights from body weight (B). Quantification of

420 serum liver enzymes for Alanine Aminotransferase (ALT) (C), Aspartate Aminotransferase (AST)

421 (D), and Alkaline Phosphatase (AP) (E) measured. Serum Albumin protein (F), serum cholesterol

422 (G) and serum triglycerides (H) quantified. Groups: ND WT n=3, ND Il23r-/- n=3, CDA-HFD WT

423 n=6, CDA-HFD Il23r-/- n=6. Data represents mean $\pm$ S.D.

424

Figure 1

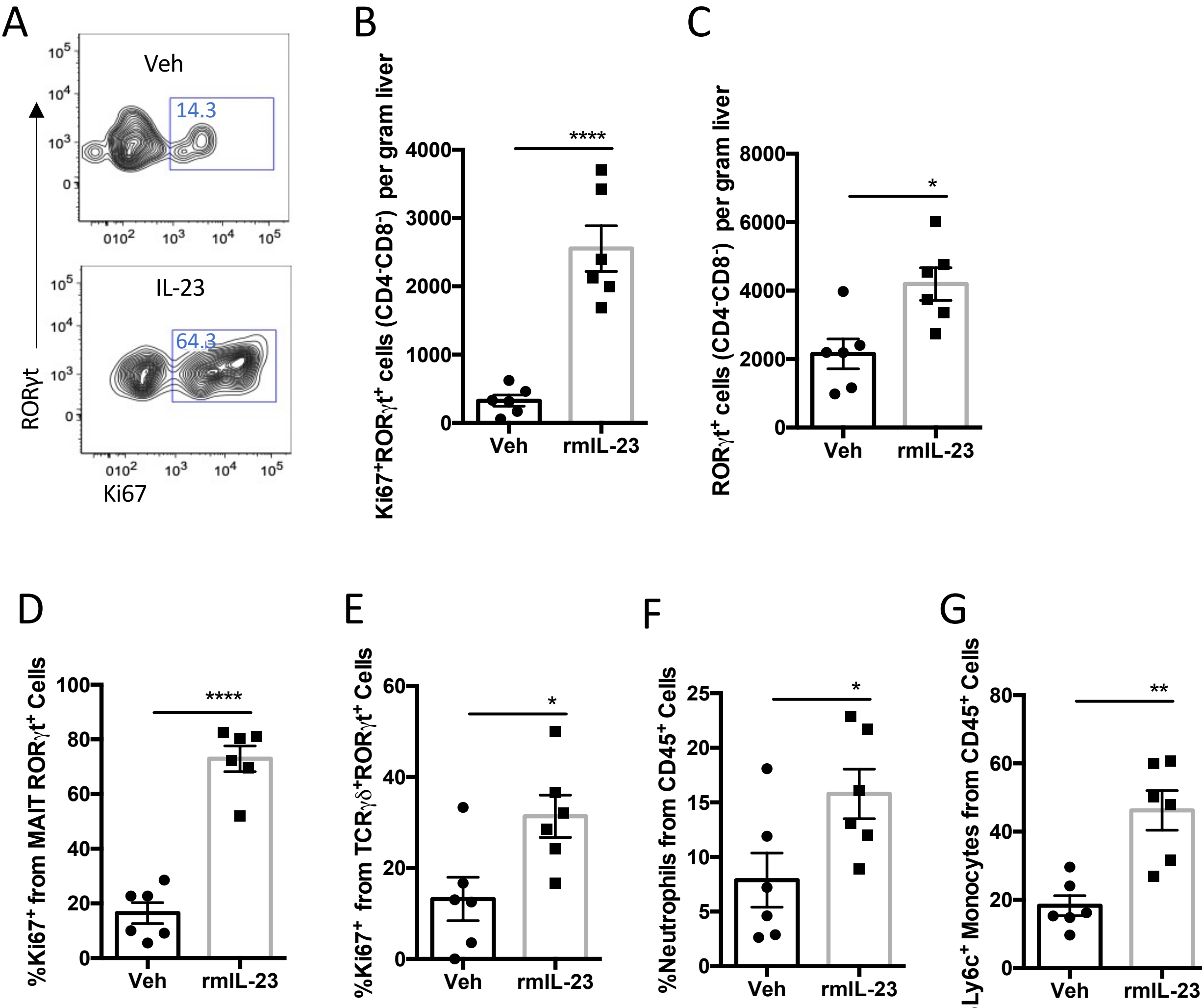
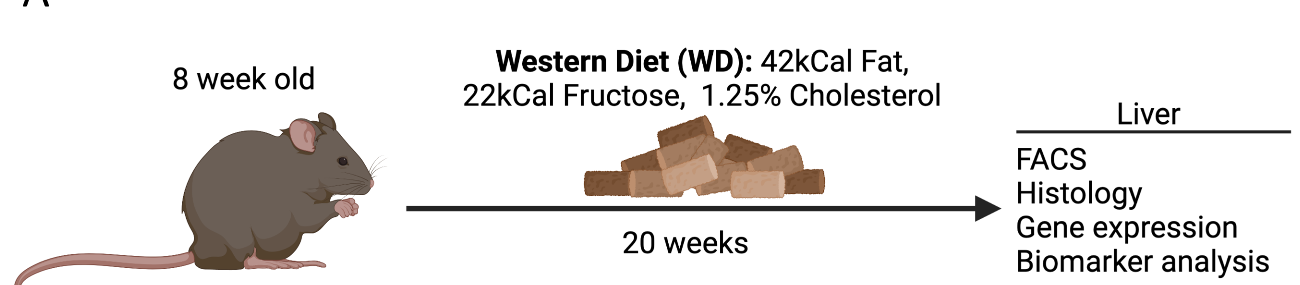


Figure 2

A



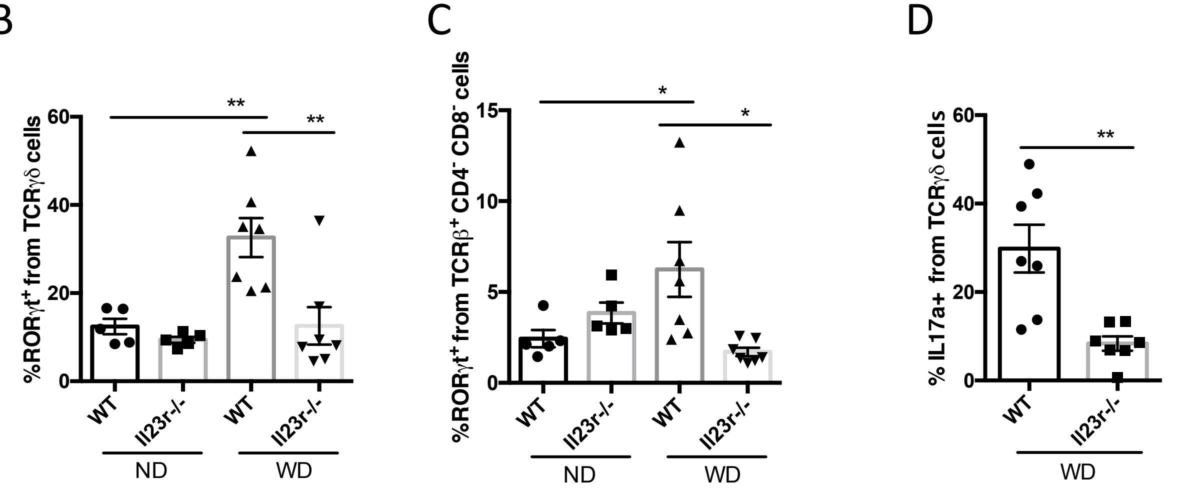


Figure 3

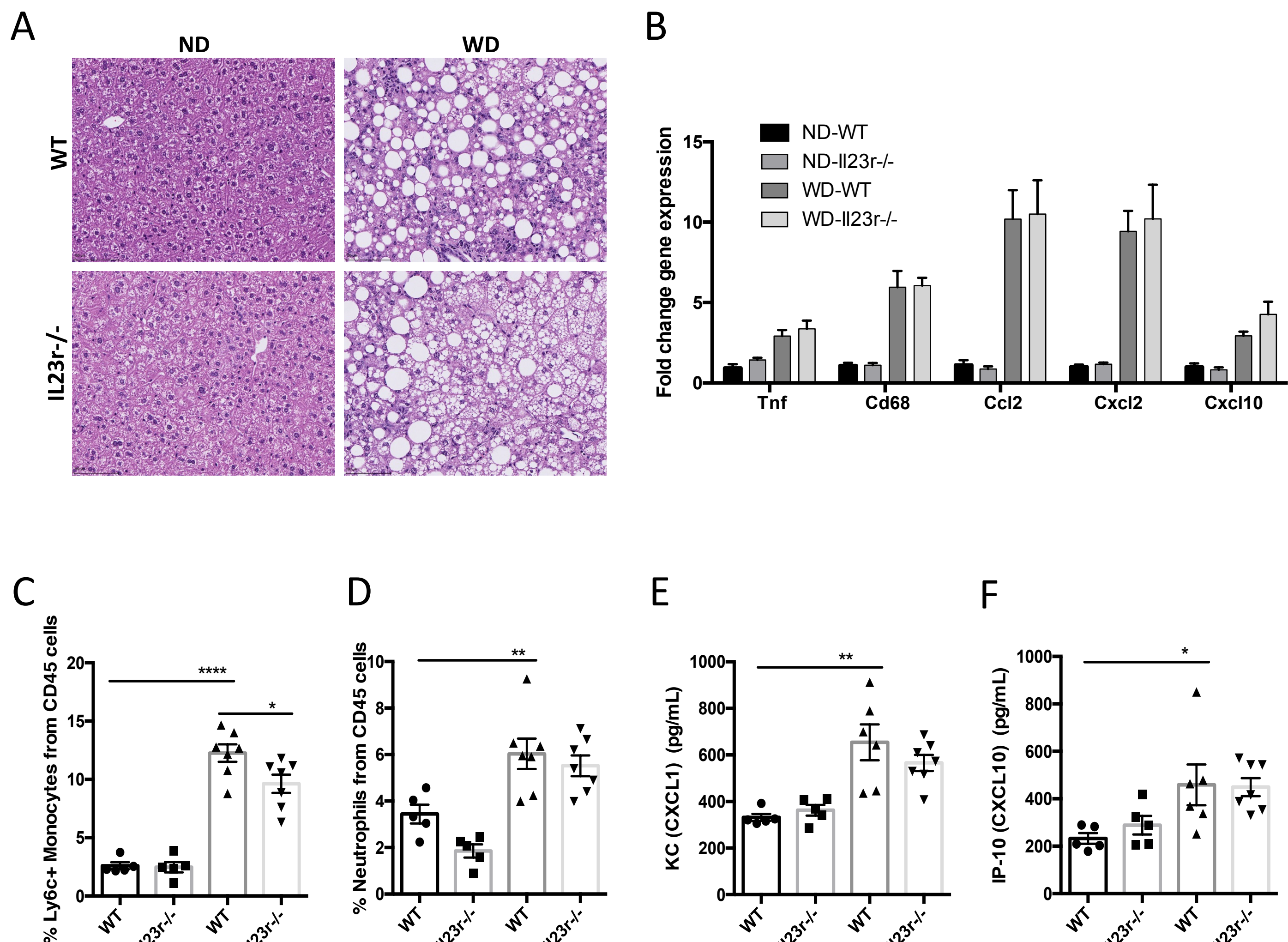


Figure 4

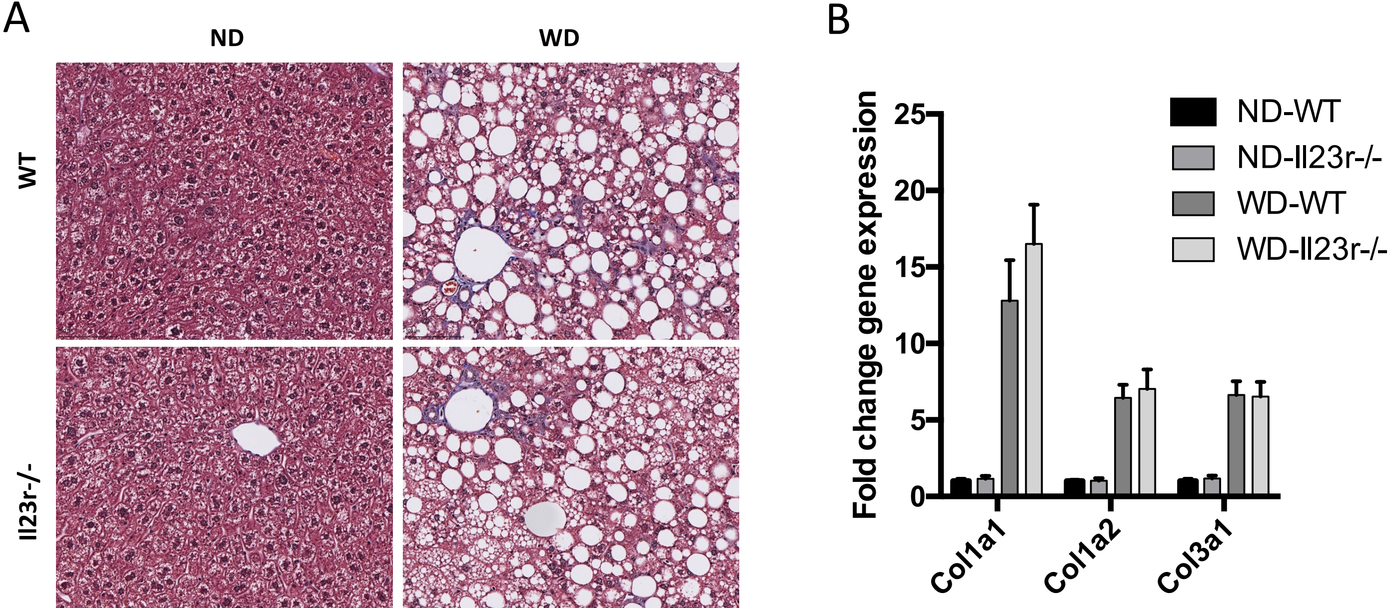


Figure 5

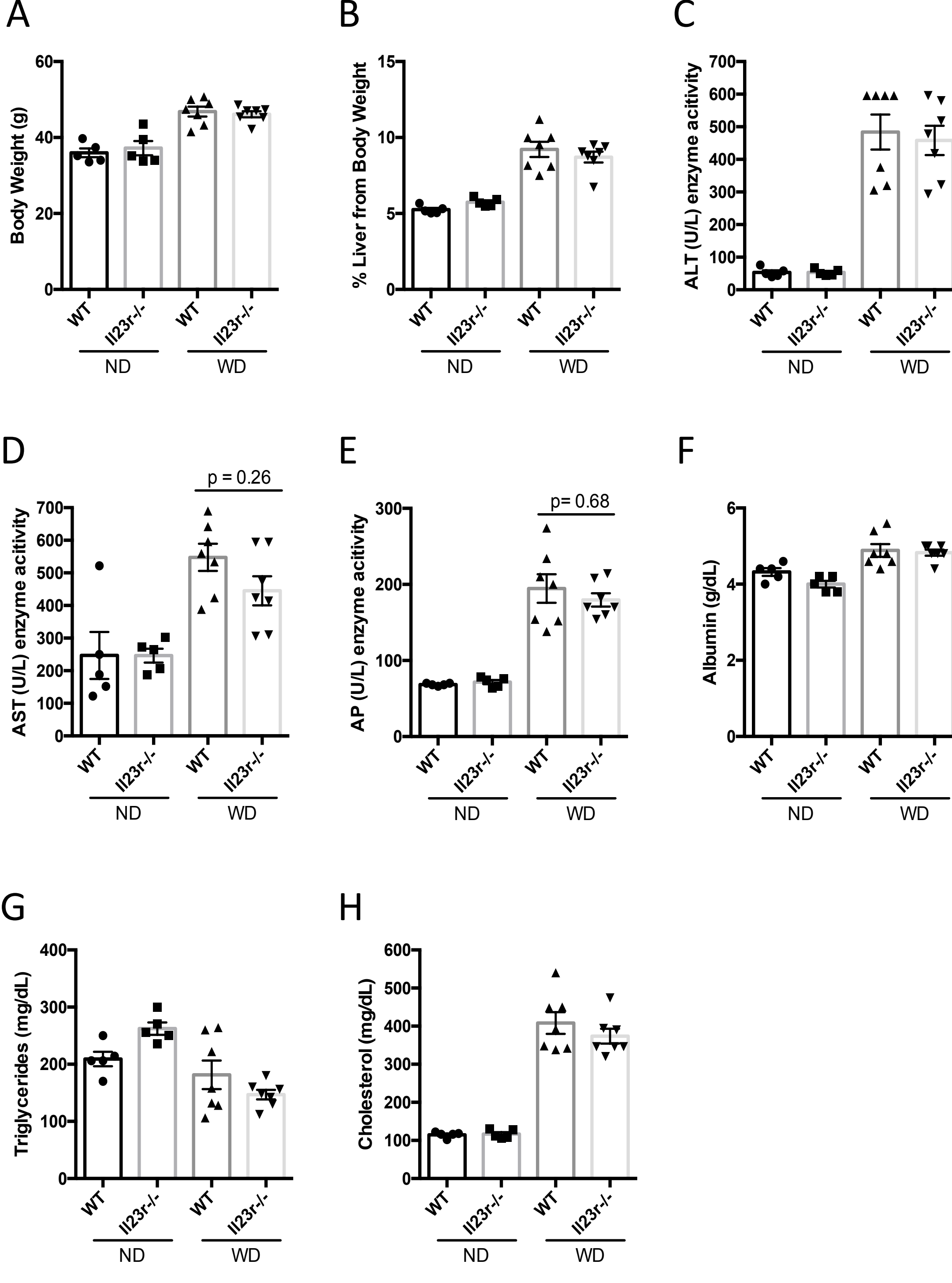


Figure 6

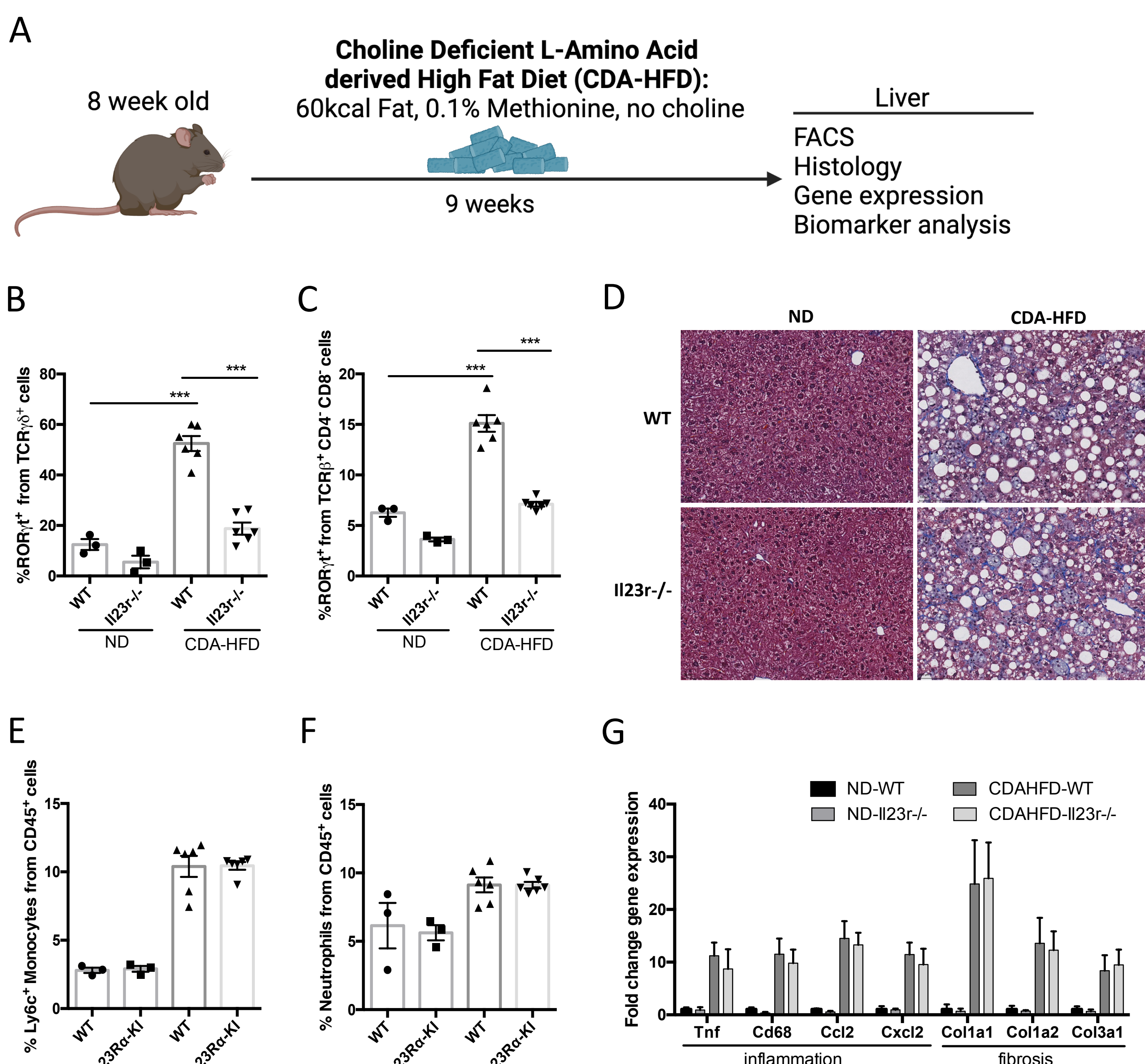


Figure 7