Intestinal IL-17R Signaling Constrains IL-18-Driven Liver Inflammation by the Regulation of Microbiome-Derived Products

Graphical Abstract

Highlights
- Disruption of intestinal IL-17RA signaling exacerbates immune-mediated hepatitis
- Enhanced disease is microbiome- and IL-18-dependent
- Intestinal IL-17RA constrains translocation of unmethylated CpG DNA to the liver
- Intestinal IL-17RA constrains hepatic FasL and type I responses by regulating IL-18

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In Brief
Castillo-dela Cruz et al. describe a unique protective role of intestinal IL-17RA in hepatitis. Disruption of intestinal IL-17RA signaling results in microbiome dysbiosis and translocation of bacterial products, specifically unmethylated CpG DNA, to the liver. This promotes IL-18 production and subsequent lymphocyte activation and cell death to exacerbate liver inflammation.
Intestinal IL-17R Signaling Constrains IL-18-Driven Liver Inflammation by the Regulation of Microbiome-Derived Products

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SUMMARY

Interleukin (IL)-17 signaling to the intestinal epithelium regulates the intestinal microbiome. Given the reported links between intestinal dysbiosis, bacterial translocation, and liver disease, we hypothesize that intestinal IL-17R signaling plays a critical role in mitigating hepatic inflammation. To test this, we study intestinal epithelium-specific IL-17RA-deficient mice in an immune-driven hepatitis model. At the naive state, these mice exhibit microbiome dysbiosis and increased translocation of bacterial products (CpG DNA), which drives liver IL-18 production. Upon disease induction, absence of enteric IL-17RA signaling exacerbates hepatitis and hepatocyte cell death. IL-18 is necessary for disease exacerbation and is associated with increased activated hepatic lymphocytes based on Ifng and Fasl expression. Thus, intestinal IL-17R regulates translocation of TLR9 ligands and constrains susceptibility to hepatitis. These data connect enteric Th17 signaling and the microbiome in hepatitis, with broader implications on the effects of impaired intestinal immunity and subsequent release of microbial products observed in other extra-intestinal pathologies.

INTRODUCTION

T helper 17 (Th17) cells play a critical role in regulating the intestinal microbiome. Within the intestine, interleukin (IL)-17 primarily signals through epithelial cells. Correspondingly, epithelial-adherent bacteria, such as segmented filamentous bacteria (SFB), both induce and are controlled by IL-17 produced by T cells (Atarashi et al., 2015; Ivanov et al., 2009; Kumar et al., 2016). Th17 cells regulate the microbiome through a variety of mechanisms including induction of anti-microbial peptides (AMPs), reactive oxygen species, and immunoglobulin A (IgA) (Belkaid and Hand, 2014; Cao et al., 2012; Kolls et al., 2008; Kumar et al., 2016). These functions act together with other Th17 cytokines to maintain intestinal barrier integrity and homeostasis (Lee et al., 2015; Schreiber et al., 2015; Shih et al., 2014; Wang et al., 2017).

There is now a growing body of research linking alterations in the intestinal microbiome to liver pathologies. For example, patients with viral and autoimmune hepatitis exhibit intestinal dysbiosis (Aly et al., 2016; Lin et al., 2015). In mouse models of these diseases, manipulation of the microbiome through antibiotics ameliorated liver inflammation (Chen et al., 2014). Given the closely related vasculature of the liver and intestine, there is a normal physiological flux of bacterial products to the liver through the portal vein draining the intestine (Macpherson et al., 2016). Indeed, many models of liver inflammation have been associated with increased bacterial translocation (Chen et al., 2014; Henao-Mejia et al., 2013; Li et al., 2017; Lin et al., 2015; Pan et al., 2012; Yan and Schnabl, 2012). While there are data showing that bacterial traffic through the hepatic circulation activates toll-like receptors (TLRs) (Chassaing et al., 2014; Henao-Mejia et al., 2013), it is not fully understood how translocation of intestinal-resident bacteria/bacterial products affect local hepatic immune cell populations. Given the importance of IL-17R in intestinal homeostasis and these reported links between liver disease and both intestinal dysbiosis and bacterial/bacterial product translocation, we hypothesized that intestinal...
IL-17R signaling may play a critical role in mitigating hepatic inflammation.

To test this, we used intestinal epithelium-specific IL-17RA knockout mice (Il17ra<sup>fl/fl</sup> x villin cre<sup>+ </sup>mice) in the concanavalin A (Con A) model of T cell-mediated hepatitis. Absence of enteric IL-17RA signaling induced commensal dysbiosis, expansion of intestinal Th17 cells, and intestinal Il18 expression. After Con A administration, Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice exhibited more severe hepatitis and increased hepatocyte cell death. Mechanistically, disease exacerbation was microbiome-dependent and associated with gram-negative bacteria. In addition, intestinal-specific knockout mice displayed increased translocation of unmethylated CpG DNA to the liver. Our data suggested that CpG DNA exacerbates liver inflammation by driving expression of hepatic IL-18 to promote interferon gamma (IFNγ) and FasL production in hepatic T cells. Thus, intestinal IL-17R regulates translocation of TLR9 ligands and constrains susceptibility to hepatic inflammation. Our studies elucidate the role of enteric Th17 signaling and the microbiome in hepatitis, with broader implications on the effects of impaired intestinal immunity and subsequent release of microbial products seen in other diseases.

**RESULTS**

**Deletion of IL-17RA in Intestinal Epithelium Exacerbates Concanavalin A Hepatitis**

To investigate how intestinal IL-17 signaling regulates liver inflammation, we treated Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice with intravenous (i.v.) with concanavalin A (Con A). Littermate Il17ra<sup>fl/fl</sup> and Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice were injected intravenously (i.v.) with concanavalin A (Con A).

(A) Serum alanine aminotransferase (ALT) at 4 h post Con A (10 mg/kg).

(B) Survival curve after 24 h post Con A (10 mg/kg).

(C) Quantification of TUNEL staining at 8 h post Con A (25 mg/kg).

(D) Representative images of TUNEL-stained liver histology at 8 h post Con A (25 mg/kg). (A, D) Data are represented as mean + SEM. (n = 5–9 mice/group) (Mann-Whitney test, Gehan-Breslow-Wilcoxon test, p<0.05*, <0.01**, <0.001***, <0.0001****).

Figure 1. Deletion of IL-17A in Intestinal Epithelium Exacerbates Concanavalin A Hepatitis

Littermate Il17ra<sup>fl/fl</sup> and Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice were injected intravenously (i.v.) with concanavalin A (Con A).

(A) Serum alanine aminotransferase (ALT) at 4 h post Con A (10 mg/kg).

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in the intestinal epithelium exacerbated disease (Figure 1). As compared to littermate Il17ra<sup>fl/fl</sup> controls, Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice exhibited elevated serum alanine aminotransferase (ALT), a marker of liver inflammation, and increased mortality rates (Figures 1A and 1B). Liver pathology revealed substantially larger patches of cell death in the hepatic parenchyma (Figure 1C). Indeed, quantification of cell death by TUNEL staining showed ~50% more cell death on average in Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice as compared to littermate floxed controls (Figure 1D).

**Exacerbated Liver Injury Is Dependent on the Intestinal Microbiota**

Given the role of Th17 cells in regulating the intestinal microbiota (Blaschitz and Raffatellu, 2010; Ivanov et al., 2009; Kumar et al., 2016), we tested whether the exacerbated liver injury was microbiome-dependent. Littermate Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice and Il17ra<sup>fl/fl</sup> controls were either cohoused or separated by genotype for 1 week prior to Con A injection. When separated, knockouts continued to demonstrate more severe disease as measured by ALT (Figure 2A). Cohousing the groups to share the intestinal microbiota between mice eliminated significant differences in ALT levels (Figure 2B), suggesting that disease exacerbation is microbiome-dependent.

IL-17 has been strongly implicated in the regulation of bacteria in the small intestine, particularly those closely related to the intestinal epithelium such as SFB (Ivanov et al., 2009; Kumar et al., 2016). To that end, we performed 16S rRNA gene sequencing on the small intestine terminal ileum of littermate Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice and floxed controls to examine changes in the microbiome due to IL-17R deficiency (Figure 2C). An outgrowth of SFB in Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice was observed in the naive state and became more pronounced after Con A (Figure 2C). There was also an outgrowth of Enterobacteriaceae in some Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice after Con A (Figure 2C). To broadly assess which bacteria may be contributing to liver disease, we treated mice with 2 different antibiotic regimens prior to Con A. Mice either received neomycin to largely target gram-negative bacteria or...
vancomycin to target SFB and other gram-positive bacteria. Based on ALT, neomycin protected mice while vancomycin had no effect, suggesting gram-negative bacteria contribute to hepatitis in this model (Figures 2D and 2E).

To characterize this further, we considered how the mechanisms by which Th17 cells regulate the microbiome are not necessarily specific to one bacterium but can affect the bacteria that generally reside close to the intestinal epithelium. Therefore, we hypothesized that there was a broad bacterial overgrowth in the gut-specific knockout mice potentially contributing to disease. Flow cytometry partnered with counting beads (Gopalakrishna et al., 2019; Palm et al., 2014; Vandeputte et al., 2017) showed increased fecal bacterial burden in Il17rafl/fl x villin cre+ mice as compared to littermate floxed controls (Figure S1A). Because transcytosis of intestinal secretory IgA is regulated by IL-17RA, IgA binding on bacteria was analyzed. Il17rafl/fl x villin cre+ mice had a substantial enrichment for IgA+ bacteria (Figures S1B and S1C). Both the general bacterial overgrowth and elevated IgA+ bacteria were depleted with neomycin (Figures S1A–S1C), suggesting that the bacteria involved in disease exacerbation were IgA+ bacteria overgrowing in the intestine in the absence of intestinal IL-17 regulation.

Next, we sought to assess how the Il17rafl/fl x villin cre+ microbiota was influencing liver disease. Because Th17 signaling is critical for mucosal barrier integrity (Blaschitz and Raffatellu, 2010; Lee et al., 2015; Shih et al., 2014), and intestinal blood drains through the portal system to the liver, it is possible that bacteria were physically translocating to the liver. Initial assessment by 16S qRT-PCR on the livers of naive Il17rafl/fl x villin cre+ mice and littermate controls showed an increased 16S rRNA gene signal in gut-specific knockout mice (Figure 2F), suggesting more bacteria or bacterial products in the liver of these mice at baseline. We were unable to grow live bacteria from the liver in both aerobic and anaerobic conditions (data not shown) and therefore used mouse TLR (mTLR) reporter cell lines to assay for bacterial products. With a focus on gram-negative bacteria, serum and liver homogenate from naive Il17rafl/fl x villin cre+ mice and littermate controls were plated on mTLR4 or mTLR9 reporter cells to measure lipopolysaccharide (LPS) and unmethylated CpG DNA levels, respectively. There were no differences in LPS levels in the serum or liver (Figures S1D and S1E). However, there was elevated CpG DNA in the liver of Il17rafl/fl x villin cre+ mice as measured by the mTLR9 reporter line (Figure 2G). In addition, we found that the CpG DNA signal in the liver was not sensitive to DNase treatment (Figure S1F), potentially implicating...
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a role for microvesicle transport of CpG. Elevated CpG DNA was not seen in serum (Figure 2H), suggesting both that the liver is successfully filtering products from entering the circulation, and CpG DNA may be signaling through TLR9 locally in the liver to influence disease. The presence of increased bacterial products without detectable live bacteria is consistent with the lack of an intestinal barrier defect in Il17rafl/fl x villin cre+ mice as measured by fluorescein isothiocyanate (FITC) dextran (Figure S1G), although the possibility of unculturable bacteria translocating to the liver cannot be ruled out. To model if elevated CpG DNA prior to disease induction exacerbates disease, wild-type C57BL/6 mice were treated with 3 doses of CpG DNA a week prior to Con A (Figure S1H). CpG DNA administration prior to Con A dramatically increased mortality rates as compared to Con A, CpG DNA, or vehicle control alone (Figure S1I).

**Intestinal IL-17RA Constrains TLR9-Induced Type I Immune Responses in the Liver**

CpG DNA and subsequent TLR9 signaling are capable of eliciting a variety of inflammatory cytokines such as IL-12, IL-6, and interferons (Gupta et al., 2006; Hartmann et al., 1999; Yi et al., 1996). To investigate which CpG-inducible cytokines may be contributing to worsened liver disease, we first wanted to establish which cytokines were elevated in Il17rafl/fl x villin cre+ mice. To employ a broad, unbiased approach, we performed single-cell RNA (scRNA) sequencing. Previous reports detailed cytokine changes at the protein level within hours of Con A injection (Wang et al., 2012). Therefore, we chose a 90-min post-injection time point to investigate transcriptional changes induced early in the model. We compared the liver transcriptome of Il17rafl/fl x villin cre+ mice against littermate Il17rafl/fl controls. Based on dimensionality reduction and upregulation of cell-specific genes (Table S1), we identified 12 distinct cell populations (Figure 3A).

Across many cell populations, Ifng was one of the significantly upregulated genes in Il17rafl/fl x villin cre+ mice (Figures 3B and 3C), while liver IL-17, other T helper cytokines, and various cytotoxic CD8+ cells were also increased (Figure 3B). Downstream IFNγ expression in Il17rafl/fl x villin cre+ mice displayed an increase in actual IFNγ+ cell number due to a 25%–30% increase in liver cellularity observed both at the naive state and 5 h post-Con A (Figure 3F). These differences were not observed in cohoused mice, suggesting these changes were microbiome-dependent (Figures S3A–S3E).

To confirm that CpG DNA can induce liver IFNγ, liver mononuclear cells from naive wild-type C57BL/6 mice were stimulated with increasing concentrations of CpG DNA or other TLR ligands. To assess whether these ligands can synergize with Con A, each TLR ligand was tested with or without Con A. CpG DNA, LPS, and lipoteichoic acid, a TLR2 ligand, all induced IFNγ responses (Figure 3G). In contrast, flagellin, a TLR5 ligand, did not induce IFNγ responses (Figure 3G). Even at low concentrations, CpG DNA induced strong IFNγ responses both in the absence and presence of Con A (Figure 3G). These data confirmed that CpG DNA is a potent inducer of IFNγ and suggested that CpG DNA may not only contribute to the elevated IFNγ observed in Il17rafl/fl x villin cre+ mice but also synergize with Con A in vivo to enhance responses and worsen disease.

To assess differences in CpG-induced IFNγ responses and determine the cellular source of IFNγ, liver mononuclear cells from Il17rafl/fl x villin cre+ mice and littermate controls were stimulated with CpG DNA ex vivo. By flow cytometry, there was already a slight increase in IFNγ+ cells in Il17rafl/fl x villin cre+ liver cells cultured in media alone (Figures 3H and 3I). This supported our hypothesis that the elevated CpG DNA at baseline may be inducing inflammatory cytokines locally. After CpG DNA

**Figure 3. Intestinal IL-17RA Constrains TLR9-Induced Type I Immune Responses in the Liver**

(A–C) Ninety minutes after i.v. concanavalin A (25 mg/kg), single-cell RNA sequencing was performed on Il17rafl/fl and Il17rafl/fl x villin cre+ liver cells enriched for mononuclear cells (n = 2 mice/group). (A) t-Distributed stochastic neighbor embedding (t-SNE) of cell type clustering based on dimensionality reduction and cell-specific gene expression. There is minor overlap between NK and T cell populations. (B) Violin plots of Ifng, Cxcl9, and Cxcl10 log-transformed gene expression in Il17rafl/fl (“Neg”) and Il17rafl/fl x villin cre (“Pos”) liver datasets. See also Figure S5 and Table S1. (C) t-SNE of Ifng expressing cells in Il17rafl/fl and Il17rafl/fl x villin cre+ datasets colored according to relative expression level. (D and E) Flow cytometry analysis of liver cells of littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice at the naive state and 5 h post Con A (25 mg/kg) (n = 2–4 mice/group). (D) Representative flow cytometry plots. (E) Number of live IFNγ+ cells, IFNγ+ TCRβ+ cells, IFNγ+ TCRβ− cells, and IFNγ+ NK Cells (gated on CD80+ TCRβ− NK1.1+). (F) Total liver cell number plotted as fold change over Il17rafl/fl (n = 3–16 mice/group). (G) Livers from wild-type C57BL/6 mice were harvested and enriched for mononuclear cells. Cells were stimulated ex vivo with varying concentrations of TLR ligands (lipoteichoic acid [LTA], flagellin [FLA], lipopolysaccharide [LPS], or CpG) + Con A (5 μg/mL). IFNγ was measured in culture supernatants at 24 h by Luminox (n = 2 replicates/condition). (H and I) Liver mononuclear cells from naive littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice were stimulated ex vivo with 1 μM CpG for 4 h plus an additional 3 h with brefeldin A and then analyzed by flow cytometry. (H) Representative fluorescence-activated cell sorting (FACS) plots. (I) Number of live IFNγ+ cells, CD4+ IFNγ+ cells (gated on live CD90+ TCRβ+), and CD8+ IFNγ+ cells (gated on live CD90+ TCRβ−) (n = 3–4 mice/group). (E–G and I) Data are represented as mean ± SEM. (unpaired t test; two-way ANOVA with multiple comparisons, multiple t tests, Wilcoxon rank-sum test). p<0.05*, <0.01**, <0.001***, <0.0001**** See also Figures S2 and S3.
treatment, the elevated IFNγ became more pronounced (Figure 3I). Much of the IFNγ came from CD4+ and CD8+ T cells (Figures 3I and S3F), although knockout mice only demonstrated significantly increased CD8+ IFNγ producers (Figure 3I). Taken together, these data suggest that in addition to having more CpG DNA in their livers at baseline, intestinal-specific knockouts demonstrate a baseline low-level elevation in IFNγ and enhanced responses to CpG DNA. This prompted the question of what factors were present in Il17rafl/fl x villin cre+ mice that were responsible for enhancing IFNγ responses. In addition, TLR9 is not highly expressed on T or NK cells, potentially implicating an intermediate factor both in vivo and ex vivo to facilitate these IFNγ responses to CpG DNA.

**Intestinal IL-17RA Constrains Hepatic and Intestinal IL-18**

scRNA sequencing also revealed increased Il18 in Il17rafl/fl x villin cre+ livers (Figures 4A and 4B). IL-18 has been implicated in many inflammatory conditions such as macrophage activation syndrome, rheumatoid arthritis, and hepatitis (Kaplanski, 2018;
We chose to investigate IL-18 further due to its established ability to enhance IFNγ production (Kaplanski, 2018; Nakamura et al., 1993).

Within the liver, bacterial products like CpG DNA have been shown to activate cells close to the hepatic vasculature, such as Kupffer cells (KCs) and hepatic stellate cells (Carpino et al., 2004; Dobashi et al., 1999; Fujita et al., 2016; Seki et al., 2001; Szabo et al., 2010). Indeed, analysis of the scRNA sequencing data showed that Il18 was expressed mainly in KCs, cholangiocytes, and to a lesser extent, a non-KC monocyte/macrophage population (Figure 4A). Consistent with the scRNA sequencing data, IL-18 was increased by transcript in the liver and protein in liver and serum of naive Il17rafl/fl x villin cre+ mice (Figures 4C–4E). Furthermore, differences in IL-18 in the liver and serum were not observed in cohoused littermate naive Il17rafl/fl and Il17rafl/fl x villin cre+ mice, suggesting the differences in IL-18 are microbiome-dependent (Figures S4A and S4B).

We also observed elevated Il18 in the small intestine of Il17rafl/fl x villin cre+ mice (Figure 4F), suggesting that in addition to local sources, the elevated liver IL-18 may also be coming from the intestine through the portal circulation. Furthermore, neomycin treatment in the drinking water (that depletes Gram-negative bacteria) decreased serum IL-18, providing additional support for not just intestinal involvement, but specifically microbiome involvement in IL-18 regulation (Figure 4G). To further support the potential contribution of intestinal IL-18 in disease exacerbation, Nrldr4+Il18bp−/− mice were treated with Con A. In these mice, IL-18 binding protein (IL-18BP), which is necessary for regulatory inhibition of IL-18, is knocked out, and the NLRC4 T337S mutation causes excess inflammasome-mediated IL-18 from the intestine (Weiss et al., 2018). After administration of Con A, these mice exhibited lethal hepatitis, while single mutant and wild-type controls survived (Figure S4C). This suggested that excess intestinal IL-18 partnered with decreased systemic inhibition of IL-18 promotes hepatitis and implies a pathogenic role for excess intestinal IL-18 in Il17rafl/fl x villin cre+ mice.

To assess whether CpG DNA plays a role in the elevated hepatic IL-18 levels, hepatic mononuclear cells were stimulated ex vivo with CpG DNA. Indeed, we observed IL-18 induction after CpG DNA stimulation (Figure 4H). Moreover, CpG-induced IFNγ was decreased ex vivo by treatment with anti-IL-18, suggesting that CpG DNA is inducing liver IFNγ in an IL-18-dependent manner (Figure 4I). To assess the functional role of IFNγ on exacerbated disease, Il17rafl/fl x villin cre+ mice were treated with anti-IFNγ prior to Con A injection. Interestingly, IFNγ blockade did not ameliorate disease (Figure S4D) despite confirmation of IFNγ neutralization by serum ELISA (Figure S4E). Because of this, we examined other downstream IL-18 targets that may be contributing to disease.

IL-18 is a pleiotropic cytokine. Beyond its ability to induce IFNγ, its range of functions include changing cell activation molecules, chemokine production, and FasL expression (Kaplanski, 2018; Tsutsui et al., 1999, 2000). We observed changes in gene expression consistent with IL-18 effects described in the literature (Bachmann et al., 2018; Eronksson et al., 2011; Gutzmer et al., 2003; Kang et al., 2007; Kojima et al., 1999; Okamoto et al., 1999; Satler et al., 2015; Subleski et al., 2007; Tsutsui et al., 1996; Weinstock et al., 2003; Yoo et al., 2005). Many of these changes were observed soon after Con A injection rather than at the naive state (Figure S5), suggesting a requirement for additional stimulation. This supports IL-18’s function as an amplifier of other cytokines (Dinarello et al., 2013). 90 min after Con A injection, there were markers of increased activation, chemokine expression, and effector function in the livers of Il17rafl/fl x villin cre+ mice (Figure 4J). Changes in these downstream Il18 targets may also be playing a role in liver pathology.

**IL-18-Induced FasL Exacerbates Liver Inflammation**

Among downstream IL-18 targets, Fas ligand (FasL) in particular, has been strongly implicated in Con A hepatitis, as knockout of either Fas or FasL is sufficient to ameliorate disease (Seino et al., 1997; Tagawa et al., 1998; Tsutsui et al., 1999). Within the liver, FasL was mainly expressed by T cells, NK cells, and NKT cells (Figure 5A). We observed increased FasL+ cells and FasL geometric mean fluorescence intensity in the liver of naive Il17rafl/fl x villin cre+ mice by flow cytometry (Figures 5B–5D). These differences were eliminated upon cohousing, suggesting these FasL changes were microbiome-dependent (Figure S6).

To test the functional role of Fasl in disease exacerbation, we treated mice with anti-Fasl 1 h prior to Con A injection. Anti-Fasl treatment ameliorated hepatitis in gut-specific knockout mice to disease severity comparable to controls based on serum ALT and TUNEL staining (Figures 5E–5G). To investigate whether IL-18 can induce liver FasL, liver mononuclear cells were stimulated ex vivo with IL-18 + anti-IFNγ and analyzed by flow cytometry (Figures 5H and 5I). While there were no significant differences between responses of naive knockouts and controls, results showed that IL-18 stimulated Fasl production in TCRβ+ cells in an IFNγ-independent manner (Figures 5H and 5I), consistent with the inability of IFNγ blockade to temper exaggerated Con A hepatitis.

**Anti-IL18 Mitigates Liver Injury in Intestinal IL-17RA-Deficient Mice**

Finally, to assess whether the elevated IL-18 in Il17rafl/fl x villin cre+ mice was contributing to worsened liver disease, we treated Il17rafl/fl x villin cre+ mice and littermate controls with anti-IL-18 or isotype control 1 day prior to Con A injection. Anti-IL-18 treatment decreased hepatitis, as measured by serum ALT, in Il17rafl/fl x villin cre+ mice to levels comparable to wild-type mice (Figure 6A). Liver pathology correspondingly showed reduced areas of cell death in the liver parenchyma (Figures 6B and 6C). Quantification of TUNEL staining confirmed that cell death in Il17rafl/fl x villin cre+ mice was substantially reduced to levels comparable to littermate floxed controls (Figure 6B). Furthermore, anti-IL-18 treatment reduced liver Ifng and Fasl transcript in Il17rafl/fl x villin cre+ mice 8 h post-Con A as measured by qRT-PCR (Figures 6D and 6E).

**DISCUSSION**

Our results provide evidence that perturbation of intestinal IL-17 signaling is sufficient to exacerbate liver inflammation. Abrogation of intestinal IL-17RA disrupted the intestinal microbiota and promoted translocation of bacterial products to the liver.
Together, this induced IL-18 production and subsequent lymphocyte activation and cell death to worsen hepatitis.

Numerous studies have implicated Th17 cells in liver inflammation. For example, both autoimmune hepatitis (AIH) and viral hepatitis patients display elevated serum IL-17 (Shin et al., 2016; Ye et al., 2010; Zhao et al., 2011). In Con A hepatitis, our lab previously showed that Il17ra<sup>−/−</sup>/C0 mice were protected from disease (Nagata et al., 2008). However, the role of Th17 cells in liver disease is complex. Here, by using intestinal epithelium-specific knockout mice, we uncouple intestinal IL-17 signaling from systemic signaling to reveal a protective role of intestinal IL-17RA in mitigating liver inflammation. Il17ra<sup>−/−</sup> x villin cre<sup>+</sup> mice do have elevations in serum IL-17A (Kumar et al., 2016) capable of mediating systemic effects. However, there are conflicting reports about the role of systemic IL-17 in Con A hepatitis (Lafdil et al., 2009; Nagata et al., 2008; Yan et al., 2012; Zenewicz et al., 2007) that we would hypothesize are due to institutional differences in the microbiome or
the contribution of other IL-17RA cytokines. This negative contribution of intestinal IL-17 relative to systemic IL-17 paralleled findings in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis. In EAE, global IL-17RA deficiency was protective (Gonzalez-García et al., 2009), while intestinal specific deficiency was detrimental (Kumar et al., 2016), indicating that intestinal IL-17 signaling and potentially control over the microbiota can have wide-ranging effects that are amplified by IL-17 signaling outside the intestine.

While reports have separately associated Th17 cells and the microbiome to liver inflammation, our study adds to the existing literature by linking these factors together in the context of liver disease (Boursier and Diehl, 2015; Chen et al., 2014; Lafdil et al., 2010; Leung and Yilmamai, 2017). Although IL-17 from multiple cellular sources (i.e., CD4 T cells, ILCs, and γδ T cells) can affect liver injury, our data suggest there is no increase in liver IL-17 within Il17rafl/fl x villin cre+ mice. Therefore, rather than IL-17 acting locally in the liver, our data suggest that disruption of intestinal IL-17RA promoted intestinal overgrowth of gram-negative, IgA+ bacteria that exacerbated liver inflammation. The presence of gram-negative bacteria exacerbating liver inflammation corroborates previous studies showing that germ-free mice and mice treated with gentamicin are protected in Con A hepatitis (Celaj et al., 2014). After Con A, Il17rafl/fl x villin cre+ mice displayed a bloom of Enterobacteriaceae, which have particularly stimulatory CpG DNA (Bouladoux et al., 2012). However, this overgrowth may simply be a consequence of inflammation as Enterobacteriaceae are known to outcompete other bacteria and bloom in inflammatory environments (Winter and Bäumler, 2014). It is also possible that we were not able to observe other changes by 16S, because an intrinsic limitation of this assay is that it does not take into account absolute bacteria counts. Therefore, the general structure of the microbiota may not appear to be changed by relative abundance of specific bacterial taxa, but changes in bacterial behavior as well as absolute amount of bacteria may contribute to disease.

In addition to alterations in the intestinal microbiome, other groups have observed increased bacterial translocation in both patients with and mouse models of liver inflammation (Chen et al., 2014; Lin et al., 2015; Pan et al., 2012). However, the downstream effects on local hepatic immune cell populations remain unclear. Here, we show that at the naive state, disruption of intestinal IL-17R signaling was sufficient to increase flux of bacterial products, specifically CpG DNA, into the liver despite no detectable functional manifestation of intestinal barrier defect. This elevated CpG DNA signal in the liver was not sensitive to DNase treatment, raising the possibility of CpG DNA transport into the liver via outer membrane vesicle (OMV) release. This

Figure 6. Anti-IL18 Mitigates Liver Injury in Intestinal Il17RA-Deficient Mice

Littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice were pre-treated with anti-IL-18 (0.5 mg/mouse) 1 day prior to IV concanavalin A (Con A) (25 mg/kg) and sacrificed at 8 h post Con A (n = 7–9 mice/group).

(A) Serum alanine aminotransferase (ALT).
(B) Quantification of TUNEL staining.
(C) Representative images of TUNEL-stained liver histology.
(D and E) Liver Ifng (D) and Fasl (E) gene expression as measured by qRT-PCR. (A–B, D–E) Data are represented as mean + SEM (one-way ANOVA with multiple comparisons test). p<0.05*, <0.01**, <0.001***, <0.0001****. Figure 6.
aligns with our data implicating gram-negative bacteria in our model, as gram-negative bacteria have been shown to produce OMVs (Schwechheimer and Kuehn, 2015). Our data and a study by Jiang et al. (2009) both demonstrated that CpG DNA worsened Con A hepatitis. There was a conflicting report detailing hepatitis attenuation after CpG DNA stimulation (Zhang et al., 2010), but we postulate this is due to differences in the CpG DNA and treatment regimen used in the experiments. In our study, we showed that CpG DNA induced IL-18-dependent liver IFNγ production from T cells, providing evidence that intestinal IL-17 signaling may play a role in constraining TLR9 induced-Type I liver responses.

Il17rafl/fl x villin cre+ mice also exhibited increased intestinal IL-18. Our data showing exacerbated hepatitis in Nirc4mut/l18bp−/− mice suggest a pathogenic role for excess intestinal IL-18. In addition, elevated intestinal IL-18 within Il17rafl/fl x villin cre+ mice is consistent with previous reports that IL-18 is produced by intestinal epithelial cells in response to IL-22 (Muñoz et al., 2015). Indeed, we have previously shown that disrupted intestinal IL-17RA signaling increased intestinal IL-22+ cells and downstream AMPs such as Reg3g (Kumar et al., 2016). Increased IL-22-driven production of AMPs may neutralize the highly toxic LPS and explain why gram-negative bacteria are implicated in disease with quantifiable differences in CpG DNA but not LPS. Interestingly, serum IL-18 was partially neutralized by intestinal IL-17RA signaling and increased intestinal IL-17RA signaling increased intestinal IL-22+ and downstream AMPs including Reg3g, thereby attenuating IL-18 dependent hepatic disease.

Excess IL-18 in Il17rafl/fl x villin cre+ mice also induced liver FaSL, increasing cell death and worsening hepatitis. This is consistent with reports implicating FaSL in Con A hepatitis. Indeed, knockout of either FaSL or FaSL ameliorated disease in this model (Seino et al., 1997; Tagawa et al., 1998; Tsutsui et al., 1999). In other model systems such as acetaminophen-induced liver injury, IL-18 has also been shown to induce Fas expression (Bachmann et al., 2018; Faggioni et al., 2001; Tsutsui et al., 1996). Here, we demonstrated that IL-18 induced FaSL in an IFNγ-independent manner. Therefore, while IL-18 is able to independently induce FaSL and IFNγ, our data suggest that the elevated IFNγ in Il17rafl/fl x villin cre+ mice was more a reflection of increased lymphocyte activation, and FaSL was the major IL-18-downstream contributor to disease exacerbation. In addition, the absence of biological or statistical differences in liver inflammation between the knockouts and controls treated with anti-IL-18 demonstrated that anti-IL-18 reduced disease in the knockout mice to levels comparable to that of controls. The lack of protection conferred to control mice suggests that IL-18 is not necessary for disease induction, but rather sufficient in excess for disease exacerbation as demonstrated in the knockout mice. Beyond our model, we established a connection between intestinal IL-17 signaling and hepatic FaSL through IL-18, potentially implicating intestinal IL-17 in the many liver diseases linked to FaSL-associated cell death including fulminant, alcoholic, and viral hepatitis as well as liver carcinoma and fibrosis (Guicciardi and Gores, 2006; Hammam et al., 2012; Pinkoski et al., 2000; Tagami et al., 2003).

Despite Con A inducing T cell-mediated hepatitis in the absence of viral infection or autoantibodies, the data within the literature suggest our work is consistent with observations in human disease. For example, AIH patients exhibit increased IFNγ and FaSL in bone marrow mononuclear cells (Tsirikoni et al., 2005), FaSL in liver tissue, and peripheral blood lymphocytes (Fox et al., 2001; Ogawa et al., 2000) compared to healthy controls. Furthermore, IFNγ in liver biopsies (Hussain et al., 1994) and serum IL-18 (Yamano et al., 2000) were both elevated and correlated with disease severity. In chronic hepatitis C, hepatic levels of IFNγ-inducible chemokines including CXCL9 and CXCL10 (Zeremski et al., 2008) and serum IL-18 (Sharma et al., 2009) were elevated and correlated with severity of liver damage. In fulminant hepatitis, there were increased circulating IFNγ+ CD8 T cells (Kimura et al., 1999) and serum IL-18 (Shinoda et al., 2006). Furthermore, a recently published report showed that excess IL-18 due to genetic IL-18BP deficiency in human patients can promote fulminant immunopathology during viral hepatitis (Belkaya et al., 2019). As such, we believe the mechanisms described in our work are potentially translatable to clinical hepatitis in patients.

Our data may also have implications on hepatic diseases and illnesses beyond the gut-liver axis in conditions with “leaky gut” and increased bacterial translocation. This study reveals potential immune consequences of the subclinical bacteremia observed in many patients. For example, in HIV/AIDS, much emphasis has been placed on leakage of LPS across the gut barrier causing wasting and chronic inflammation (Brenchley et al., 2006). Our data suggest that extra-intestinal TLR9 ligand dissemination is regulated by intestinal IL-17 signaling and may therefore be another underlying mechanism in HIV/AIDS liver dysfunction (Chamroonkul and Bansal, 2019). Taken together, the connection our data established between intestinal Th17 cells, the microbiome, and hepatic immune signaling elucidate therapeutic avenues to explore to treat hepatitis and other extra-intestinal diseases.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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  - Animal treatments
  - Alanine aminotransferase quantification
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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-IL-18          | Genentech | N/A        |
| Anti-mouse IFNγ     | BioXCell | Cat#BE0055-A025mg; RRID: AB_1107694 |
| Anti-mouse FasL      | BioLegend | Cat#93971; RRID: AB_313281 |
| IgG Isotype Control | BioLegend | Cat#92257, RRID: AB_11203529 |
| BV786 Anti-mouse CD4 | BD Biosciences | Cat#563727; RRID: AB_2728707 |
| APC Anti-mouse IFNγ | eBioscience | Cat#17-7311-82; RRID: AB_469504 |
| APC780 Anti-mouse TCRβ | eBioscience | Cat#47-5961-82; RRID: AB_1272173 |
| BV421 Anti-mouse NK1.1 | BD Biosciences | Cat#562921; RRID: AB_2728688 |
| BV395 Anti-mouse CD3 | BD Biosciences | Cat#563565; RRID: AB_2738278 |
| PCP-Cy5 Rat-Anti-Mouse CD3 Molecular Complex | BD Biosciences | Cat#560527; RRID: AB_1727463 |
| BV605 Anti-Mouse CD90.2 | BD Biosciences | Cat#740334; RRID: AB_2740067 |
| PE Anti-Mouse FasL   | BioLegend | Cat#106660; RRID: AB_313279 |
| PE-Cy7 Anti-mouse CD8 | eBiosciences | Cat#25-0083-82; RRID: AB_11218494 |
| Anti-Mouse CD16/CD32 | eBiosciences | Cat#14-0161-86; RRID: AB_467135 |
| PE anti-mouse IgA    | eBiosciences | Cat#12-4204-81; RRID: AB_465916 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Concanavalin A from Canavalia ensiformis (jack bean) type IV | Sigma-Aldrich | Cat#C2010 |
| Ultrapure LPS, E. coli 0111:B4 | Invivogen | Cat#tlr-3pelps |
| Purified LTA from S. aureus | Invivogen | Cat#tlr-psla |
| TLR9 Agonist - Stimulatory CpG ODN, Class C, Human / mouse | Invivogen | Cat#tlr-2395 |
| Ultrapure flagellin from B. subtilis | Invivogen | Cat#tlr-psfsf |
| E. coli dsDNA | Invivogen | Cat#tlr-ecdna |
| Critical Commercial Assays | | |
| ALT Activity Assay | Sigma-Aldrich | Cat#MAK052 |
| ApopTag® Peroxidase In Situ Apoptosis Detection Kit | Millipore Sigma | Cat#S7100 |
| Mouse IFN-γ ELISA | BioLegend | Cat#430804 |
| Mouse IL-18 ELISA | Invitrogen | Cat# BM5618-3; RRID: AB_2575692 |
| Pierce BCA Protein Assay Kit | Thermo Scientific | Cat# 23225 |
| Chromium™ Single Cell 3’ Gel Bead Kit v2 | 10x Genomics | Cat# PN-120235 |
| MinElute PCR Purification Kit | QIAGEN | Cat#28004 |
| Purelink PCR Purification Kit | Life Technologies | Cat#K310001 |
| MiSeq Reagent Kit v2 (500-cycles) | Illumina | Cat#MS-102-2003 |
| LIVE/DEAD Fixable Aqua Dead Cell Stain Kit | Invitrogen | Cat#L34966 |
| MILLIPOLEX Mouse Th17 Magnetic Bead Panel | Millipore Sigma | Cat#MTH17MAG-47K |
| Cytokine & Chemokine 36-Plex Mouse Procarta Plex Panel 1A | Thermo Fisher Scientific-Affymetrix | Cat#EPX360-26092-901; RRID: AB_2576123 |
| Deposited Data | | |
| Liver Single Cell RNA sequencing | This Paper | GEO: GSE128284 |
| 16S rRNA sequencing | This Paper | SRA BioProject: PRJNA526489 |
| Terminal ileum RNA sequencing | Kumar et al., 2016 | SRA: SRP069071 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

#### Experimental Models: Cell Lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEK-Blue mTLR9 cells | Invivogen | Cat#hkb-mtlr9 |
| HEK-Dual mTLR4 cells | Invivogen | Cat#hkd-mtlr4ni |

#### Experimental Models: Organisms/Strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: C57BL/6      | Taconic Biosciences | C57BL/6NTac |
| Mouse: Il17ra<sup>fl/fl</sup> | Kumar et al., 2016 | MGI:5907986 |
| Mouse: Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> | Kumar et al., 2016 | N/A |
| Mouse: Nlrc4<sup>mut</sup> | Weiss et al., 2018 | N/A |
| Mouse: Nlrc4<sup>mut</sup>Il18bp<sup>−/−</sup> | Courtesy of collaborator, Dr. Scott Canna | N/A |
| Mouse: Il18bp<sup>−/−</sup> | UC Davis KOMP Repository | MGI:1333800 |

#### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primer for 16S rDNA: Forward (Eubact Uni340FP) | Croswell et al., 2009; Salzman et al., 2010; Kumar et al., 2016 | N/A |
| Primer: mouse Hprt | Integrated DNA Technologies | Cat# Mm.PT.39a.22214828 |
| Primer: mouse Ifng | Applied Biosystems (Thermo Fisher Scientific) | Cat#Mm01168134_m1 |
| Primer: mouse Il18 | Applied Biosystems (ThermoFisher Scientific) | Cat#Mm00434226_m1 |
| Primer: mouse FasL | Applied Biosystems (ThermoFisher Scientific) | Cat#Mm00438864_m1 |

#### Software and Algorithms

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Cell Ranger version 2.1.1 | 10x Genomics | https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger |
| Seurat suite version 2.2.1 | Satija Lab | https://satijalab.org/seurat/ |
| Loupe Browser | 10x Genomics | https://support.10xgenomics.com/single-cell-gene-expression/software/visualization/latest/what-is-loupe-cell-browser |
| Prism | Graphpad | https://www.graphpad.com/scientific-software/prism/ |
| Mothur | Schloss et al., 2009 | N/A |
| R package | Tarabichi et al., 2015 | N/A |
| FlowJo version 10 | Flowjo | https://www.flowjo.com |
| ImageJ | NIH | https://imagej.nih.gov/ij/index.html |

#### Other

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Trizol | Life Technologies | Cat#15596-018 |
| Trizol LS | Life Technologies | Cat#10296010 |
| iScript Reverse Transcription Supermix | Bio-RAD | Cat#1708841 |
| SSoFast Supermix | Bio-RAD | Cat#1725281 |
| SYBR Green Supermix | Bio-RAD | Cat#1708880 |
| Roche cComplete Protease Inhibitor Cocktail | Sigma Aldrich | SKU: 11697498001 |
| QUANTI-Blue:Alkaline phosphatase detection medium - Powder | Invivogen | Cat#rep-qb1 |
| IMDM, GlutaMAX Supplement | Thermo Fisher Scientific | Cat#31980097 |
| FBS | Thermo Fisher Scientific | Cat#A3160502 |
| Penicillin-Streptomycin-Glutamine (100X) | Thermo Fisher Scientific | Cat#10378016 |
| Collagenase | Sigma Aldrich | Cat#C5138-500MG |
| DNase I | Sigma Aldrich | Cat#1284932001 |

(Continued on next page)
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jay K. Kolls (jkolls1@tulane.edu). Distribution of the anti-IL-18 reagent is restricted under an MTA with Genentech.

**Experimental Model and Subject Details**

**Mice**

All mouse work was performed in accordance with the Institutional Animal Care and Use Committees (IACUC) and relevant guidelines at the University of Pittsburgh, School of Medicine. C57BL/6 mice were obtained from Taconic Biosciences (Germantown, NY). Nlrp4<sup>-/-</sup> and Nlrp4<sup>-/-</sup> mice were obtained from Dr. Scott Canna at the UPMC Children’s Hospital of Pittsburgh. Il17ra<sup>-/-</sup> and Il17ra<sup>-/-</sup> x villin cre<sup>+</sup> mice were obtained from Dr. Scott Canna at the UPMC Children’s Hospital of Pittsburgh.

Il17ra<sup>-/-</sup> and Il17ra<sup>-/-</sup> x villin cre<sup>+</sup> mice were generated at the UPMC Children’s Hospital of Pittsburgh by crossing Il17ra<sup>-/-</sup> mice to Il17ra<sup>-/-</sup> x villin cre<sup>+</sup> mice. Both male and female age-matched mice from 6-10 weeks of age were used for all experiments. The aforementioned breeding strategy allowed for controls and knockout mice within each experiment to be littermates. Littermate age-matched males and females were randomly assigned to experimental groups. Both males and females were used within each group in order to account for sex-differences while maintaining littermate controls and sufficient n for statistical power. All mice were housed in pathogen-free conditions at the UPMC Children’s Hospital of Pittsburgh.

**In vitro and ex vivo cultures**

Mouse TLR9 and TLR4 reporter cells (HEK-blue mTLR9 and HEK-dual mTLR4 reporter cells) were obtained from Invivogen and maintained according to manufacturer’s instructions. Sex of both cell lines: female. Ex vivo stimulation of liver cells: Livers from 6-10-week-old naive Il17ra<sup>-/-</sup> mice and Il17ra<sup>-/-</sup> x villin cre<sup>+</sup> mice were harvested and enriched for mononuclear cells by Percoll gradient. In addition to detailed experiment-specific stimuli, cells were maintained at 37°C in Iscove’s Modified Dulbecco’s Medium (IMDM) with GlutaMAX Supplement (GIBCO), 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin and streptomycin, and 0.3mg/mL of L-glutamine.

**Experimental Models**

Concanavalin A (Con A) hepatitis was induced using concanavalin A from *Canavalia ensiformis* (jack bean) type IV (Sigma).

**Method Details**

**Animal treatments**

C57BL/6, Il17ra<sup>-/-</sup>, and Il17ra<sup>-/-</sup> x villin cre<sup>+</sup> mice were injected with 10mg/kg or 25mg/kg Con A intravenously (IV) via tail vein. For antibiotic studies, mice were treated with either five days of 1g/L neomycin or 14 days of 0.5g/L vancomycin in the drinking water ad libitum prior to Con A injection and remained on antibiotics throughout the hepatitis model. For CpG DNA pre-treatments, Class C CpG (Invivogen) was injected 3x at 2.5mg/kg intraperitoneally (IP) prior to 10mg/kg IV Con A. For IFNγ blockade, anti-IFNγ (BioXCell, Clone XMG1.2) was injected IP at 0.5mg/mouse two hours prior to Con A injection. For FasL inhibition, mice were injected with anti-FasL (BioLegend, Clone MFL3) at 250-500 μg/mouse IV into the retro-orbital sinus one hour prior to 25mg/kg Con A injection. For IL-18 blockade, anti-IL-18 (Genentech) was injected IP at 0.5mg/mouse one day prior to 25mg/kg IV Con A injection.

**Alanine aminotransferase quantification**

Alanine aminotransferase (ALT) was measured in the serum of mice using the Vitros DT60 II chemistry system (Ortho-Clinical Diagnostics, Inc.) (Zheng et al., 2016) or ALT Activity Assay (Sigma) per manufacturer’s instructions. Method of ALT measurement was consistent within experiments.
**TUNEL Staining**
Liver tissues used for TUNEL staining were immediately fixed in 4% paraformaldehyde for 24-72 hours, washed 3x in PBS, and stored in 70% ethanol prior to paraffin embedding. Following paraffin embedding, slides were stained using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit according to manufacturer’s instructions.

**Cohousing studies**
Littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice were either kept cohoused or separated for one week prior to Con A injection and remained in assigned housing conditions throughout hepatitis model.

**qRT-PCR and RNA Sequencing**
Livers and intestines from naive 6-10-week-old littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice were homogenized in Trizol buffer (Life Technologies). Total RNA extraction was performed according to Trizol manufacturer’s instructions. RNA was transcribed into cDNA using iScript reagent (Bio-RAD) according to manufacturer’s instructions.

For qRT-PCR, SYBR Green supermix (Bio-RAD) was used for analysis of small subunit ribosomal RNA gene (16S rRNA) expression. 16S primers included: forward: ACTCCTACGGGAGGCAGCAGT, reverse: ATTACCGCGGCTGCTGGC (Croswell et al., 2009; Kumar et al., 2016; Salzman et al., 2010). SsoFast supermix (Bio-RAD) was used for qRT-PCR analysis with primers for mouse Hprt (Integrated DNA Technologies), Ifng (Applied Biosystems), and Fasl (Applied Biosystems). Expression of all genes was normalized relative to housekeeping gene mouse Hprt. Reaction: 95°C for 3 minutes, 49 cycles at 95°C for 10 s (s) and 60°C for 30 s. SYBR Green reactions also had an additional melt curve at the end of the reaction above: 60°C for 5 s with +0.5°C increase every cycle up to 95°C.

Terminal ileum bulk RNA sequencing data was sourced from dataset we previously published (Kumar et al., 2016). See previous manuscript for detailed methods.

**Single Cell RNA Sequencing**
Livers from 6-week-old littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice were harvested at the naive state or ninety minutes post 25mg/kg IV Con A injection. Single cell suspensions were isolated and enriched for mononuclear cells via Percoll gradient. Briefly, livers were collected in IMDM with GlutaMax (GIBCO) supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine (“complete media”). Livers were minced into small pieces and digested in neat IMDM with 1mg/mL collagenase and 0.2mg/mL DNase at 37°C for 30 minutes with shaking. Cell suspension was further homogenized by flowing through an 18G needle in a 3mL syringe followed by filtering through a 70 µm filter. Following a wash in complete media, mononuclear cells were enriched using a 70%/30% percoll gradient. Cells were washed 2x in complete media and resuspended for downstream applications.

For single cell RNA sequencing library preparation, liver cells were then separated into mini-reaction “partitions” or Gel bead in emulsion (GEMs) formed by oil micro-droplets, each containing a gel bead and a cell, by the Chromium instrument (10X Genomics). The reaction mixture/emulsion with captured and barcoded mRNAs were removed from the Chromium instrument followed by reverse transcription. The cDNA samples were fragmented and amplified per 10X protocol. The libraries were then purified, quantified, and sequenced on an Illumina NextSeq 550. Analysis was performed using the pipeline Cell Ranger developed by 10X Genomics as well as Seurat.

**16S rRNA Gene Sequencing**
Littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice were sacrificed at the naive state or 8 hours post Con A injection. Terminal ileum RNA was isolated using Trizol (Life Technologies) and transcribed to cDNA using the iScript Reverse Transcription Supermix (Bio-Rad), both according to manufacturer’s instructions. Extracted DNA was PCR amplified using the method/primers of Caporaso et al. (2012) and the Q5 HS High-Fidelity polymerase (NEB). Four microliters of each sample were amplified in a 25 µL PCR reaction with barcoded V4 16S primers. Cycle conditions were 98°C for 30 s, then 25 cycles of 98°C for 10 s, 57°C for 30 s, 72°C for 30 s, with a final extension step of 72°C for 2 min. Reactions were purified with AMPure XP beads (Beckman) at a 0.8:1 ratio (beads:DNA) to remove primer-dimers. Eluted DNA was quantitated on a Qubit fluorimeter (Life Technologies). Sample pooling was performed on ice by combining 20ng of each purified band. For negative controls and poorly performing samples, 20 µL of each sample was used. The sample pool was first purified/concentrated with the MinElute PCR purification kit. Next, two-sided AMPure XP bead purification was used at 0.8:1 (left-side) and 0.61:1 (right-side) ratios to remove small and large contaminants, respectively. A final cleanup in the Purelink PCR Purification Kit (Life Technologies) was performed to insure removal of all AMPure XP beads. The final, purified pool was quantitated in triplicate on the Qubit fluorimeter prior to sequencing.

Sequencing pool preparation was as per Illumina’s recommendations, with an added incubation at 95°C for 2 minutes immediately following the initial dilution to 20 picomolar. The pool was then diluted to a final concentration of 6 pM + 15% PhiX control. Paired-end sequencing was done on an Illumina MiSeq platform using a MiSeq Reagent kit v2 (500 cycles).

**mTLR reporter assays**
For mTLR4 and mTLR9 reporter cell assays, cells were grown and maintained according to manufacturer’s instructions (Invivogen). For the assay, cells were stimulated with serum or liver homogenate of naive littermate Il17rafl/fl and Il17rafl/fl x villin cre+ mice. For the
liver homogenate, livers were homogenized in PBS plus protease inhibitor (Roche). BCA Protein Assay (Pierce) was performed according to manufacturer’s instructions to measure total protein concentration. Dilutions were performed in PBS plus protease inhibitor to normalize concentrations between samples prior to reporter assay. SEAP levels were measured using QUANTI-Blue detection media (Invitrogen). Assays were performed according to the manufacturer’s instructions.

For DNase treatment of liver homogenate, livers were harvested, homogenized in PBS plus protease inhibitors, and diluted to normalize total protein concentration as described above. An aliquot of liver homogenate was treated with DNase I from bovine pancreas (Sigma) reconstituted in PBS + MgCl to activate enzyme per manufacturer’s instructions. Vehicle control treatment was PBS + MgCl without DNase. Liver homogenate plus DNase or vehicle control were incubated at 37°C with shaking for 30 minutes. Digested samples were then plated on mTLR9 reporter cell line as described above. Positive control to ensure DNase treatment efficacy was E. coli dsDNA (Invitrogen). Negative controls were nuclelease free water (Thermo Fisher) and nuclease-resistant Class C CpG (Invitrogen).

Flow cytometry

Liver single cell suspensions were isolated and enriched for mononuclear cells from C57BL/6 mice or littermate Il17ra<sup>−/−</sup> and Il17ra<sup>+/+</sup> x villin cre<sup>+</sup> mice as described above in Single Cell RNA Sequencing. For staining, cells were washed in HBSS. Surface and live/dead stains were performed in 50 μL-75 μL in a 96 well round bottom plate in the dark on ice for 20 minutes. Cells were washed 2.5x in cold FACS Buffer (0.5% FBS/0.01% Na3/PBS). Cells were then fixed using BD Cyto-fix and incubated in the dark on ice for 20 minutes. If no further staining was required, cells were washed 2x in FACS buffer, resuspended in PBS or FACS buffer, and analyzed using the BD Fortessa flow cytometer. If additional intracellular stain analysis was required, cells were washed 1x in BD Perm/Wash. 50-75 μL of intracellular stain cocktail made in BD Perm/Wash was then added to the cells and incubated in the dark on ice for 45 minutes. Cells were then washed 2x in BD Perm Wash, 1x in FACS buffer, and resuspended in PBS or FACS buffer for analysis on the BD Fortessa flow cytometer. Data analysis was performed on FlowJo. Cell number was quantified using the Nexcelom Cellometer Auto 2000. Flow cytometry antibodies used included: Live/Dead fixable aqua dead cell stain (Invivogen) (1:500 dilution), BV786 Anti-mouse CD4 (BD Clone RM4-5) (1:200 dilution), APC Anti-mouse IFNγ (eBiosciences Clone XMG1.2) (1:200 dilution), Apc780 Anti-mouse TCRβ (BD Clone H57-597) (1:200 dilution), BV421 Anti-mouse NK1.1 (BD clone PK136) (1:100 dilution), BV395 Anti-mouse CD3 (BD Clone 145-2C11) (1:200 dilution), PCP-Cy5 Rat-Anti-Mouse CD3 Molecular Complex (BD clone 17A2) (1:200 dilution), BV605 Anti-Mouse CD90.2 (BD clone 30-H12) (1:400 dilution), PE Anti-Mouse FasL (Biolegend Clone MFL3) (1:200 dilution), PE-Cy7 Anti-mouse CD8 (BD Clone 145-2C11) (1:200 dilution), BV421 Anti-mouse NK1.1 (BD clone PK136) (1:100 dilution), BV395 Anti-mouse CD3 (BD Clone 145-2C11) (1:200 dilution), PCP-Cy5 Rat-Anti-Mouse CD3 Molecular Complex (BD clone 17A2) (1:200 dilution), BV605 Anti-Mouse CD90.2 (BD clone 30-H12) (1:400 dilution), PE Anti-Mouse FasL (Biolegend Clone MFL3) (1:200 dilution), PE-Cy7 Anti-mouse CD8 (Invitrogen clone eBioH35-7.2) (1:400 dilution), Anti-Mouse CD16/CD32 (eBioscience Clone 93).

Fecal Bacterial Flow Cytometry

Protocol based on previously published works (Gopalakrishna et al., 2019; Palm et al., 2014; Vandeputte et al., 2017). Briefly, fecal matter was collected and weighed. 1ml of sterile PBS was added to the fecal content and homogenized by vortex and pipetting. Stool suspension was then passed through a 40-micron strainer into a 50ml conical tube. 10 μL of stool was added to a round-bottom 96-well plate for IgA staining. Plated stool was washed 2.5x in BAC-FACS buffer (filtered 1%BSA in PBS) at 4000rpm for 5 minutes at 4°C. Stool was then stained in BAC-FACS buffer using Hoechst stain (1:1000 dilution) (Life Technologies), normal rat serum (1:5 dilution) (Thermo Fisher), and anti-mouse IgA PE or isotype control (1:500 dilution) (eBioscience clone mA-6E1). Samples were stained for one hour on ice and then washed 2.5x with 100 μL BAC-FACS buffer. Samples were reconstituted in BAC-FACS buffer, and 10 μL of Accu-Check counting beads (Thermo Fisher) were added to each. Samples were analyzed by flow cytometry on the BD Fortessa cytometer. Bacterial calculations using Accu-Check beads (Thermo Fisher) were done using manufacturer’s instructions.

Ex vivo TLR ligand stimulation

Liver single cell suspensions were isolated and enriched for mononuclear cells from C57BL/6 mice or littermate Il17ra<sup>−/−</sup> and Il17ra<sup>+/+</sup> x villin cre<sup>+</sup> mice as described above in Single Cell RNA Sequencing. Cells were then resuspended in IMDM with GlutaMax (GIBCO) supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine (“complete media”) and plated at a concentration of 5 × 10<sup>5</sup> or 1 × 10<sup>6</sup> cells per well in a 96-well round bottom plate. Cell number was kept consistent within experiments. Cells were then stimulated with various conditions at 37°C for the detailed incubation times. Lipoteichoic acid, flagellin, lipopolysaccharide, and CpG were all attained from Invivogen and used at 10ng/mL-1 μg/mL or 1 μM-5 μM. Exact concentrations are detailed within each figure. During stimulations with TLR ligands ± Concanavalin A, 5μg/mL of Concanavalin A (Sigma) was used. Supernatants were harvested at 24 hours and analyzed for IFNγ and IL-18 levels by ELISA or Luminex. For downstream flow cytometry analysis, brefeldin A (BD) was added for 3 hours after 4 hours of 1 μM CpG stimulation. Cells were then stained and fixed for flow cytometry analysis as described above in Flow Cytometry.

ELISA and Luminex Assays

Cytokines from serum, liver homogenate, and cell culture supernatants were measured using the following ELISA or Luminex kits according to the manufacturer’s instructions: Mouse-IFNγ ELISA MAX Kit (BioLegend), MILLIPLEX Mouse Th17 Magnetic Bead Panel (Millipore Sigma), Cytokine & Chemokine 36-Plex Mouse Procarta Plex Panel 1A (Thermo Fisher Scientific-Affymetrix), and IL-18 Mouse ELISA Kit (Invitrogen).
FITC Dextran Assay for Intestinal Permeability

Four hours prior to FITC dextran gavage, water bottles were removed from the mouse cages. FITC-dextran (4kDa, Sigma) was dissolved in PBS at a concentration of 100 mg/ml and administered to each mouse at 44mg/100 g body weight by oral gavage. Mice were euthanized after 4 hours, and blood was collected immediately after via cardiac puncture. Serum was isolated from blood samples. For analysis, serum was diluted with an equal volume of PBS. 100 μL of diluted serum was added to a 96-well microplate in duplicate. Concentration of FITC in serum was determined by fluorescence spectroscopy. The plate was read at an excitation of 485 nm (20 nm band width) and an emission wavelength of 528 nm (20 nm band width). Serially diluted FITC-dextran (0, 125, 250, 500, 1,000, 2,000, 4,000, 6,000, 8,000 ng/ml) was used as a reference standard to calculate serum concentrations. Serum from mice gavaged with saline instead of FITC-dextran was used to determine background.

QUANTIFICATION AND STATISTICAL ANALYSIS

TUNEL Image Quantification

To quantify TUNEL staining, five images spanning the width of the liver slice were taken at 10x magnification. Images were analyzed using ImageJ. Briefly, images were deconvoluted to isolate and analyze only the TUNEL diaminobenzidine (DAB) staining. A threshold of the TUNEL DAB stain was determined to minimize background staining (i.e., vascular endothelial cells, erythrocytes). Identical threshold was applied to all samples per experiment. Using the “measure” feature on ImageJ, TUNEL+ staining was then quantified as the percent of the total image area that was above the set color threshold. To ensure that focal batches of cell death throughout the liver were accounted for, the % area TUNEL+ of all five images were averaged to determine the % Area TUNEL+ per mouse.

Single Cell RNA Sequencing Analysis

Following sequencing described above, we used Cell Ranger version 2.1.1 (10x Genomics) to process raw sequencing data and Seurat suite version 2.2.1 for downstream analysis. Filtering was performed to remove multiplets and broken cells, and non-relevant sources of variation were regressed out. Variable genes were determined by iterative selection based on the dispersion versus average expression of the gene. For clustering, principal component analysis was performed for dimension reduction. Top 10 principal components (PCs) were selected by using a permutation-based test implemented in Seurat and passed to t-SNE for visualization of clusters.

Bulk Intestinal RNA Sequencing Analysis

Data presented is sourced from the dataset we previously published (Kumar et al., 2016). See previous manuscript for detailed statistical analysis.

16S rRNA Gene Sequencing Analysis

Sequence read quality control and classifications were completed using the Center for Medicine and the Microbiome in-house read processing and classification pipeline. The read processing pipeline applied low complexity filtering (NCBI dustmasker), QV trimming, sequence adaptor trimming and primer trimming modules. Sequences with both forward and reverse read directions passing read processing metrics were assembled using the make.contig command from Mothur (Schloss et al., 2009). Mated reads were further screened to limit overlap mismatch proportion (< 0.2), limit N’s allowed (4), and enforce a minimal overlap of 25bp. Merged sequences were classified with a Mothur-dependent in-house pipeline that combines OTU generation and taxonomic classifications using the RDP/Silva classifier and includes chimera screening, clustering and taxonomic classification. The sample taxonomic profile was subsequently represented as a matrix with dimensions: number of samples x number of taxonomic units for compositional analysis with an in-house pipeline which incorporated statistical modules and graphics using the R package (Tarabichi et al., 2015).

Statistical Tests

Statistical tests used are indicated in the figure legends. To compare differences between two groups, student-t test or non-parametric Mann-Whitney test was used depending on the distribution of the data. When comparing one variable in three or more groups, one-way ANOVA with multiple comparisons was used. When comparing multiple variables among two groups, two-way ANOVA with multiple comparisons or multiple t tests per row was used. GraphPad Prism software was used to analyze experimental groups. For single cell RNA sequencing, statistical analysis was based on the non-parametric Wilcoxon rank sum test. For all data, statistically significant was defined as p < 0.05. The degree of statistical significance was defined as: p < 0.05*, < 0.01**, < 0.001***, < 0.0001****.

Analysis Software

GraphPad Prism was used for statistical analysis described above. ImageJ was used for histology analysis. Seurat, Cell Ranger, and Loupe Browser were used for single cell RNA sequencing analysis.
DATA AND CODE AVAILABILITY

The accession number for the 16S rRNA sequencing data reported in this paper is SRA BioProject: PRJNA526489. The accession number for the liver single cell RNA sequencing data reported in this paper is GEO: GSE128284 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128284). The accession number for the original terminal ileum RNA sequencing data (Kumar et al., 2016) is SRA: SRP069071.
Supplemental Information

Intestinal IL-17R Signaling Constrains
IL-18-Driven Liver Inflammation
by the Regulation of Microbiome-Derived Products

Patricia Castillo-dela Cruz, Alanna G. Wanek, Pawan Kumar, Xiaojing An, Waleed Elsegeiny, William Horne, Adam Fitch, Ansen H.P. Burr, Kathyayini P. Gopalakrishna, Kong Chen, Barbara A. Methé, Scott W. Canna, Timothy W. Hand, and Jay K. Kolls
SUPPLEMENTAL INFORMATION

Figure S1. Related to Figure 2. (A) Fecal bacterial counts from naïve II17raβ/β and II17raβ/β x villin cre+ mice treated with neomycin or H2O control as measured via flow cytometry. (B) Representative FACS plots of fecal bacteria stained with isotype or IgA (Gated by FSC, SSC, and Hoechst+). (C) Quantification of IgA unbound (IgA-) and IgA bound (IgA+) bacterial counts with and without neomycin treatment in II17raβ/β and II17raβ/β x villin cre+ mice. Data are representative of multiple experiments. (A-C) n = 4-5 mice/group. (D-E) Serum (D) and liver homogenate (E) from naïve littermate II17raβ/β and II17raβ/β x villin cre+ mice were plated on mTLR4 SEAP reporter cells. Absorbance of supernatants was measured and represented as ratio over null/unstimulated cells. (n =7-14 mice/group). (F) Liver homogenate from naïve littermate II17raβ/β and II17raβ/β x villin cre+ mice were treated with DNase or vehicle control, then plated on mTLR9 SEAP reporter cells. (n=4-8 mice/group). Controls: E. coli dsDNA, Nuclease-resistant CpG DNA, and nuclease free H2O. Absorbance of supernatants was measured and represented as ratio over null/unstimulated cells. (G) Serum FITC Dextran (4kDA) concentration in control saline-gavaged mice (n=2) and FITC dextran-gavaged II17raβ/β and II17raβ/β x villin cre+ mice (n=3 mice/group). (H-I) Experimental schematic of treatment regimen (H) and survival curve (I) of wildtype C57BL/6 mice pretreated with IP 2.5mg/mL CpG 3x prior to IV Con A injection (10mg/mL). (n=4-5 mice/group). (A, C, D-G) Data are represented as mean ± SEM. p<0.05*, <0.01**, <0.001***, <0.0001**** (Unpaired t-test, Two Way ANOVA with multiple comparisons test, Log Rank (Mantel-Cox) Test)
Figure S2. Related to Figure 3. (A) Liver Il17a expression in II17ra<sup>fl/fl</sup> and II17ra<sup>fl/fl</sup> x villin cre<sup>+ </sup>mice at the naïve state and 5h-post Con A as measured by qRT-PCR (n = 3-4 mice/group). (B) IL-17 in liver homogenate of naïve II17ra<sup>fl/fl</sup> and II17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice was measured by Luminex (n = 4-8 mice/group). (C) Ninety minutes after IV Con A (25mg/kg), single cell RNA sequencing was performed on II17ra<sup>fl/fl</sup> ("Neg") and II17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> ("Pos") liver cells enriched for mononuclear cells. (n=2 mice/group). TSNE of Il17 expressing cells colored according to relative expression level and violin plot of Il17a expression (normalized, log-transformed). (D-F) Percent (D), number (E), and MFI (F) of live IL-17+ cells in the livers II17ra<sup>fl/fl</sup> and II17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice at the naïve state and 5h-post Con A (n = 2-4 mice/group). (G) Ninety minutes after IV Con A (25mg/kg), single cell RNA sequencing was performed on II17ra<sup>fl/fl</sup> ("Neg") and II17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> ("Pos") liver cells enriched for mononuclear cells--Violin plots of genes of interest (n=2 mice/group).
Figure S3.
Related to Figure 3. (A-E) Livers from cohoused Il17ra<sup>−/−</sup> and Il17ra<sup>−/−</sup> x villin cre<sup>+</sup> mice at the naïve state and 5h-post Con A were harvested, enriched for mononuclear cells, and analyzed via flow cytometry (n = 3-4 mice/group). (A) Number of IFNγ+ cells (gated on live cells). (B) Number of IFNγ+ TCRβ- cells (gated on live CD90+). (C) Representative FACS plots. (D) Number of IFNγ+ TCRβ+ cells (gated on live CD90+). (E) Total liver cell count per gram of tissue. (F) Livers from naive littermate Il17ra<sup>−/−</sup> and Il17ra<sup>−/−</sup> x villin cre<sup>+</sup> mice were harvested and enriched for mononuclear cells. Cells were stimulated <i>ex vivo</i> with 1μM CpG for 4 hours plus an additional 3 hours with brefeldin A, and then analyzed by flow cytometry. Percent and actual cell number of IFNγ+ cells by cell type (gated on live IFNγ+ cells) (n=3-4 mice/group). Data are represented as mean ± SEM. p<0.05*, <0.01**, <0.001***, <0.0001**** (Multiple T tests per row).
Figure S4.
Related to Figure 4. (A-B) IL-18 in liver homogenate (A) and serum (B) of naïve cohoused Il17ra<sup>-/-</sup> and Il17ra<sup>-/-</sup> x villin cre<sup>+<sup> mice (n=3-4 mice/group). (C) Con A (25mg/kg) was injected intravenously into wildtype (WT) B6 mice, NLRC4 mutants (Nlrc4<sup>mut</sup>), IL-18 binding protein knockouts (Il18bp<sup>-/-</sup>), and mice having both the NLRC4 mutation and IL-18BP deficiency (Nlrc4<sup>mut</sup>Il18bp<sup>-/-</sup>). Survival Curve (n = 3-10 mice/group). (D-E) Con A (25mg/kg) was injected IV into Il17ra<sup>-/-</sup> x villin cre<sup>+</sup> mice treated with anti-IFNγ or control two hours prior to Con A injection. (D) Serum ALT at 8h post injection (n = 9-13 mice/group). (E) Serum IFNγ at 8h post injection as measured by ELISA (n = 3-5 mice/group). (A-B, D-E) Data are represented as mean + SEM. p<0.05*, <0.01**, <0.001***, <0.0001**** (Unpaired T Test, Log Rank (Mantel-Cox) Test).
**Figure S5.** Related to Figures 3-4. Single cell RNA sequencing was performed on naïve *Il17ra*+/− ("Neg") and *Il17ra*+/− x villin cre+ ("Pos") liver cells enriched for mononuclear cells. (n=2 mice/group). Violin plots of Ifng, Cxcl9, and Cxcl10 (A), Il18 (B), and various effector function, chemokine, and activation related genes (C) displayed as normalized, log-transformed expression level. p<0.05*, <0.01**, <0.001***, <0.0001**** (Wilcoxon Sum Rank Test).
Figure S6. 
Related to Figure 5. Livers from Il17ra<sup>fl/fl</sup> and Il17ra<sup>fl/fl</sup> x villin cre<sup>+</sup> mice at the naïve state and 5h-post Con A were harvested, enriched for mononuclear cells, and analyzed via flow cytometry (n = 3-4 mice/group). Percent (A) and number (B) of FasL<sup>+</sup> cells (gated on live cells). (C) Representative FACS plots. (D) FasL gMFI (gated on live FasL<sup>+</sup> cells). (E) Representative histograms. (A-B, D) Data are represented as mean + SEM.
Table S1. Single cell RNAseq cluster gene lists. Related to Figure 3-4. Cell-type specific gene lists (bold) and top 50 most significantly differentially expressed genes per cluster.

| Cluster | Genes | Genes |
|---|---|---|
| 1 | Col1a1 | Clec4f |
| 2 | Col1a2 | Spic |
| 3 | Acta1 | Mcaro |
| 4 | Tagln | Vsig4 |
| 5 | Col1a3 | Cplv |
| 6 | Rbp1 | Sparc |
| 7 | Acta2 | Col14a1 |
| 8 | Ccl19 | Meg3 |
| 9 | Tagln | Pcolce |
| 10 | Ccl17 | Ifil1 |
| 11 | Alb | Col15a1 |
| 12 | Hbb-bt | Hba-a2 |

Dpt | Clec4f | Col14a1 |
Clcl9 | Clec4f | Col15a1 |
Mfap4 | Clq | Col15a2 |
Gsn | Follr2 | Cplv |
Mmp2 | Vsig4 | Cpx |
Htr3 | Clqa | Cpx |
Lum | Fcan | Cpx |
Sparcl1 | Acta2 | Cpx |
Col1a2 | Adgre1 | Cpx |
Col1a1 | C1qb | Cpx |
Sfrp1 | Wdf1c17 | Cpx |
Col3a1 | Csfr1 | Cpx |
Clec3b | Cfp | Cpx |
Tagln | D68 | Cpx |
Gpx3 | Oas1 | Cpx |
Myh11 | Ctsck | Cpx |
Col6a2 | Lst1 | Cpx |
Mustn1 | Slc40a1 | Cpx |
L0x1 | Plira | Cpx |
Tnfaip6 | Mpeg1 | Cpx |
Ccl11 | C5ar1 | Cpx |
Tpm2 | Ctsck | Cpx |
Col6a1 | Cxcl13 | Cpx |
Cxcl12 | Bcl2a1a | Cpx |
Myh7 | Myc | Cpx |
Smc2 | Slc3 | Cpx |
Thbs1 | Mmp13 | Cpx |
Mylk | Illb | Cpx |
Fst1l | Cyyb | Cpx |
Serpin1 | Tnfaip2 | Cpx |
Eln | Fgr3 | Cpx |
Mgp | Rfp2 | Cpx |
Lhhfa | Cxcl2a | Cpx |
Dcn | Illr1n | Cpx |
Igfbp3 | Bcl2a1b | Cpx |
Tppp3 | Bcl2a1b | Cpx |
Ptx3 | Spp1 | Cpx |
Col1a4 | Arg2 | Cpx |
Ogn | Kcd12 | Cpx |
Ili1a1 | Clec12a | Cpx |
Meg3 | Clec4na | Cpx |
Cdx4 | Fpr1 | Cpx |
Nbl1 | Tyrbop | Cpx |
Adm | Cic7 | Cpx |
Poc1d4 | Clec4p | Cpx |

| Cluster Type | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Type | Col1a1 | Col1a2 | Acta1 | Tagln | Col1a3 | Rbp1 | Sparc | | | | | |
| Genes | Clec4f | Spic | Mcaro | Vsig4 | Cplv | Cbf | | | | | | |
| Genes | Clq | Tff2 | Sgcb3a1 | Cxyl2 | Fxyd2 | Defb1 | | | | | | |
| Genes | Col14a1 | Col15a2 | Ifil1 | Pcolce | Rbp1 | | | | | | | |