

Interleukin-33-Dependent Innate Lymphoid Cells Mediate Hepatic Fibrosis

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SUMMARY

Liver fibrosis is a consequence of chronic liver diseases and thus a major cause of mortality and morbidity. Clinical evidence and animal studies suggest that local tissue homeostasis is disturbed due to immunological responses to chronic hepatocellular stress. Poorly defined stress-associated inflammatory networks are thought to mediate gradual accumulation of extracellular-matrix components, ultimately leading to fibrosis and liver failure. Here we have reported that hepatic expression of interleukin-33 (IL-33) was both required and sufficient for severe hepatic fibrosis *in vivo*. We have demonstrated that IL-33's profibrotic effects related to activation and expansion of liver resident innate lymphoid cells (ILC2). We identified ILC2-derived IL-13, acting through type-II IL-4 receptor-dependent signaling via the transcription factor STAT6 and hepatic stellate-cell activation, as a critical downstream cytokine of IL-33-dependent pathologic tissue remodeling and fibrosis. Our data reveal key immunological networks implicated in hepatic fibrosis and support the concept of modulation of IL-33 bioactivity for therapeutic purposes.

INTRODUCTION

Liver fibrosis—a widespread consequence of chronic liver diseases that encompass infection-related hepatic fibrosis (e.g., hepatitis C virus infections, Schistosomiasis) metabolic-type fibrosis (e.g., steatohepatitis), and biliary-type fibrosis (e.g., primary sclerosing cholangitis)—is a major cause of mortality and morbidity. It develops as a result of chronic inflammatory responses to persistent hepatocellular stress, which finally culminates in pathologic tissue remodeling and increased deposits of extracellular matrix (ECM) proteins (Battaller and Brenner, 2005). Later stages of progressive liver fibrosis are characterized by fundamental changes in liver architecture, which predispose to organ failure and hepatocellular carcinomas.

Hepatic stellate cells (HSCs) are viewed as a predominant cellular source of ECM components during fibrosis. Injury-associated immunological processes supporting transdifferentiation of quiescent HSC into fibrogenic myofibroblasts in the course of liver injury were shown to be particularly important for fibrosis (Friedman, 2010). The inflammatory response associated with liver injury and fibrosis is a dynamic process that involves intrahepatic accumulation of immune cell subsets. Those are attracted to and activated in the liver by specific patterns of chemokines and cytokines produced by liver-resident cells. Chemokines and cytokines like transforming growth factor- β (TGF- β), IL-6, PDGF, and IL-1 have been implicated in HSC function *in vitro* and *in vivo* (Seki et al., 2009; Wasmuth et al., 2010; Weiler-Normann et al., 2007). However, many of the early events in liver fibrosis, including the exact nature of infiltrating pathogenic immune cell subsets and key molecular mediators, which support local inflammation and activation of HSC remain undefined (Friedman, 2008).

Recently, interleukin-33 (IL-33), an IL-1-related cytokine, has been implicated in inflammatory conditions in animal models (Haraldsen et al., 2009; Schmitz et al., 2005; Xu et al., 2008). In addition, IL-33 has been suggested to possess profibrotic properties in skin and lung (Rankin et al., 2010; Yanaba et al., 2011). IL-33 might be actively secreted from cells by yet-undefined pathways, but passive release from necrotic cells is reported to be a dominant mechanism for abundance of extracellular IL-33 *in vivo* (Cayrol and Girard, 2009; Lüthi et al., 2009). Therefore, it is proposed that IL-33 might be considered as a damage-associated molecular pattern (DAMP) that contributes to tissue injury responses as well as inflammatory responses (Palmer and Gabay, 2011). Cytokine-like activity of extracellular IL-33 is mediated through a heteromeric cell-surface receptor consisting of the ubiquitous IL-1R associated protein (IL-1R3) and the more selectively expressed IL-33 receptor (ST2, IL-1R4) (Chackerian et al., 2007; Palmer et al., 2008). Recently, phenotypically similar populations of innate lymphoid cells (ILC) that express ST2 and respond to IL-33 by production of the T helper-2 (Th2) cell-associated cytokines IL-4, IL-5, and IL-13 have been described (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010b). Furthermore, these cells (ILC2) have been shown to be a major *in vivo* source of IL-9 (Wilhelm et al., 2011) and intrinsic expressions of the transcription factors ROR α and GATA3 have been shown to be important for the generation of at least

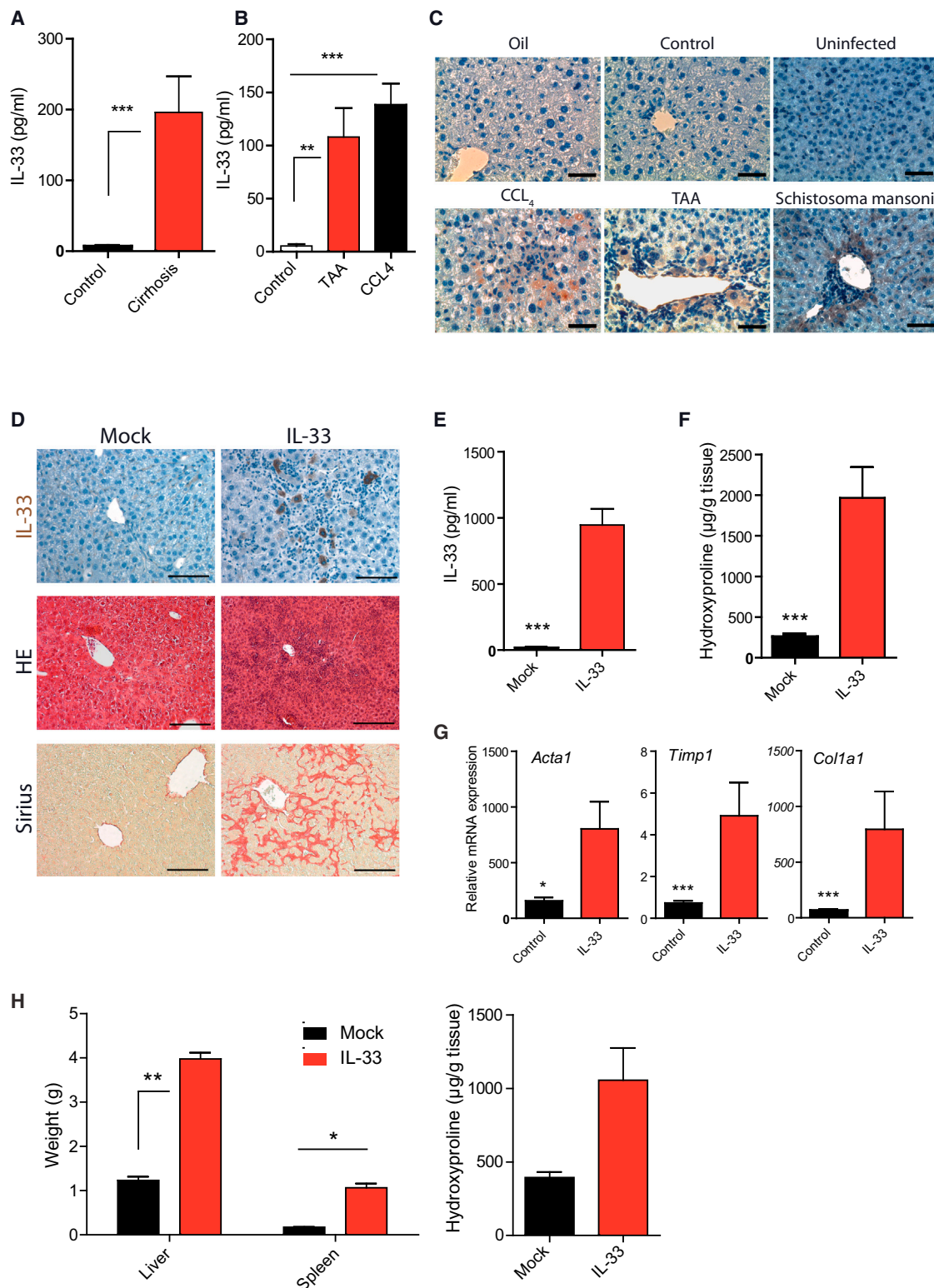


Figure 1. IL-33 Is Upregulated in Hepatic Fibrosis

(A) Serum IL-33 (n = 11 per group; ***p < 0.001) in healthy controls or cirrhosis patients was determined by ELISA.

(B) Serum IL-33 protein concentrations in control mice or mice with TAA or CCL₄ induced liver fibrosis. (n = 5 per group; **p < 0.01; ***p < 0.001) were determined.

(C) Representative images of liver sections from CCL₄, TAA, *S. mansoni*-infected and control-treated mice stained for IL-33 by immunohistochemistry. Scale bars represent 50 μ m.

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some of these ILC2 populations (Wong et al., 2012; Hoyler et al., 2012). In mice and humans, ILC2 are primarily found in lymphoid tissues associated with barrier surfaces and a critical role of ILC2 and ILC2-derived cytokines has been suggested in the context of host protection against helminths and viral infections (Chang et al., 2011; Mjösberg et al., 2011; Monticelli et al., 2011).

Here we have identified IL-33 as key mediator of hepatic fibrosis in vivo. Our data suggest that IL-33 is released in response to chronic hepatocellular stress and that extracellular IL-33, via ST2-dependent signaling, leads to accumulation and activation of ILC2 in the liver. Activated hepatic ILC2 produce IL-13, which in turn triggers activation and transdifferentiation of HSC in an IL-4R α - and STAT6 transcription-factor-dependent fashion. These findings characterize molecular and cellular networks implicated in hepatic fibrosis and highlight the role of IL-33 at the apex of the profibrotic cascade. In addition, a pathogenic capacity of ILC2 in the context of the tissue damage response is unveiled. Finally, our results validate the idea of therapeutic modulation of IL-33-dependent networks in hepatic inflammation and fibrosis.

RESULTS

IL-33 Expression Is Elevated in Hepatic Fibrosis and IL-33 Is Sufficient to Drive Excess ECM Deposition in the Liver

Previous studies suggest a role for IL-33 in the context of Th2 cell-associated inflammation and fibrosis of skin and lung (Palmer and Gabay, 2011; Rankin et al., 2010). To address its association with development of human fibrotic liver disease, we evaluated IL-33 serum concentrations in a cohort of patients with liver cirrhosis. Significantly higher serum amounts of IL-33 were observed in patients compared to controls (Figure 1A). In rodent models of hepatic fibrosis, chronic hepatocellular stress is mimicked by repeated application of hepatotoxic chemicals. Hence, we next evaluated the expression of IL-33 in mice in which liver fibrosis was experimentally induced in two independent models by repeated administration of Thioacetamide (TAA) or Carbon tetrachloride (CCl₄). In mice treated with either chemical, IL-33 serum concentrations were significantly increased (Figure 1B), and increased hepatic IL-33 protein expression was demonstrated by immunohistochemical staining (Figure 1C). Furthermore, hepatic IL-33 protein expression was strongly increased in a mouse model of chronic Schistosomiasis, an infection predisposing mice and humans to severe hepatic inflammation and fibrosis (Figure 1C). In addition, IL-33 messenger RNA (mRNA) expression in the liver was elevated by TAA and CCl₄ treatment (data not shown). No differential IL-33 expression was found in other tissues of CCl₄ or TAA

subjected mice suggesting that increased serum IL-33 is unlikely to originate from nonhepatic cells in these models (data not shown).

In order to establish whether release of IL-33 from liver cells is sufficient for induction of fibrosis, we generated a vector encoding a secreted version of IL-33 (mclIL-33) controlled by liver-specific regulatory elements and took advantage of the technology of hydrodynamic delivery (HD) for sustained in vivo transduction of hepatocytes with IL-33 (Liu et al., 1999) (see Figure S1A available online). Our data confirmed that IL-33 was overexpressed in hepatocytes of IL-33 vector-injected but not control vector-injected mice (Figure 1D). Similarly, IL-33 protein was detected in serum (Figure 1E). Other studies have demonstrated that rIL-33 can induce systemic expression of IL-13 in vivo (Rankin et al., 2010; Schmitz et al., 2005). At the IL-33 doses used for HD in our studies, no serum IL-13 was detectable (data not shown), suggesting that liver-specific rather than systemic effects of IL-33 were observed.

Four weeks after HD, IL-33 vector-injected but not control vector-injected animals showed excessive immune cell infiltrates and increased hepatic collagen as evident by hematoxylin and eosin (H&E) and Sirius Red staining, respectively (Figure 1D). A significant increase in total hepatic collagen was confirmed by applying the quantitative hydroxyproline assay to harvested liver tissues (Figure 1F). Treatment with IL-33 also induced gene expression of fibrogenic markers *Acta1*, *Timp1*, and *Col1a1* (Figure 1G). No increase in hepatic collagen was observed upon injection of the same dose of IL-33 vector into IL-33R (ST2)-deficient mice, confirming that ST2 is required for IL-33-mediated hepatic fibrosis (Figure S1B).

Moreover, *S. mansoni* dependent liver damage strongly increased upon mclIL-33 mediated hepatic IL-33 overexpression (Figure 1H), and such treated mice display premature death due to severe disease (data not shown).

To further analyze the functional role of the IL-33 pathway in hepatic fibrosis in vivo, we expressed biologically active IL-33 in livers of mice. We generated a conditional transgenic line that allowed tissue- or cell-type-specific overexpression of IL-33 by Cre recombinase-mediated deletion of an EGFP-Stop cassette (eGFP^{STOPfllox}/IL33 mice; Figure S1C). We induced hepatic IL-33 expression in eGFP^{STOPfllox}/IL33 mice by HD of a Cre-expression plasmid (Figure S1D). Examination of livers of these mice 4 weeks later revealed that hepatic IL-33 expression led to profound collagen depositions and fibrosis (Figure S1D).

IL-33 Deficiency Ameliorates Experimental Fibrosis In Vivo

We next investigated whether IL-33 deficiency affects development of hepatic fibrosis in vivo; fibrosis was induced in *IL33*^{-/-}

(D) We HD injected 2.5 μ g of mclIL-33 expression or control vector (mock) into mice. Mice were analyzed 4 weeks later. Liver sections were stained for IL-33 protein, with HE or, for visualization of collagen, with Sirius Red.

(E) IL-33 serum concentrations in mclIL-33 treated mice (** p < 0.001) were determined by ELISA. Scale bars represent 100 μ m (IL-33 staining) or 200 μ m (HE and Sirius Red).

(F) Total hepatic collagen of indicated mice was quantified by hydroxyproline assay (n = 9–10 per group; *** p < 0.001).

(G) mRNAs of selected fibrosis-associated genes in liver total RNA were quantified by qPCR (n = 9–10 per group; *** p < 0.001; * p < 0.05). Data are representative of at least two different experiments with similar results.

(H) CD1 mice infected with ~30 *S. mansoni* cercaria were HD injected with mclIL-33 at week 3 after infection. Three weeks later, mice were sacrificed and organ weights were determined. Hepatic collagen content was determined by hydroxyproline quantification (n = 5 per group; ** p < 0.01; * p < 0.05). Data are representative of two different experiments with similar results. Error bars indicate SEM. See also Figure S1.

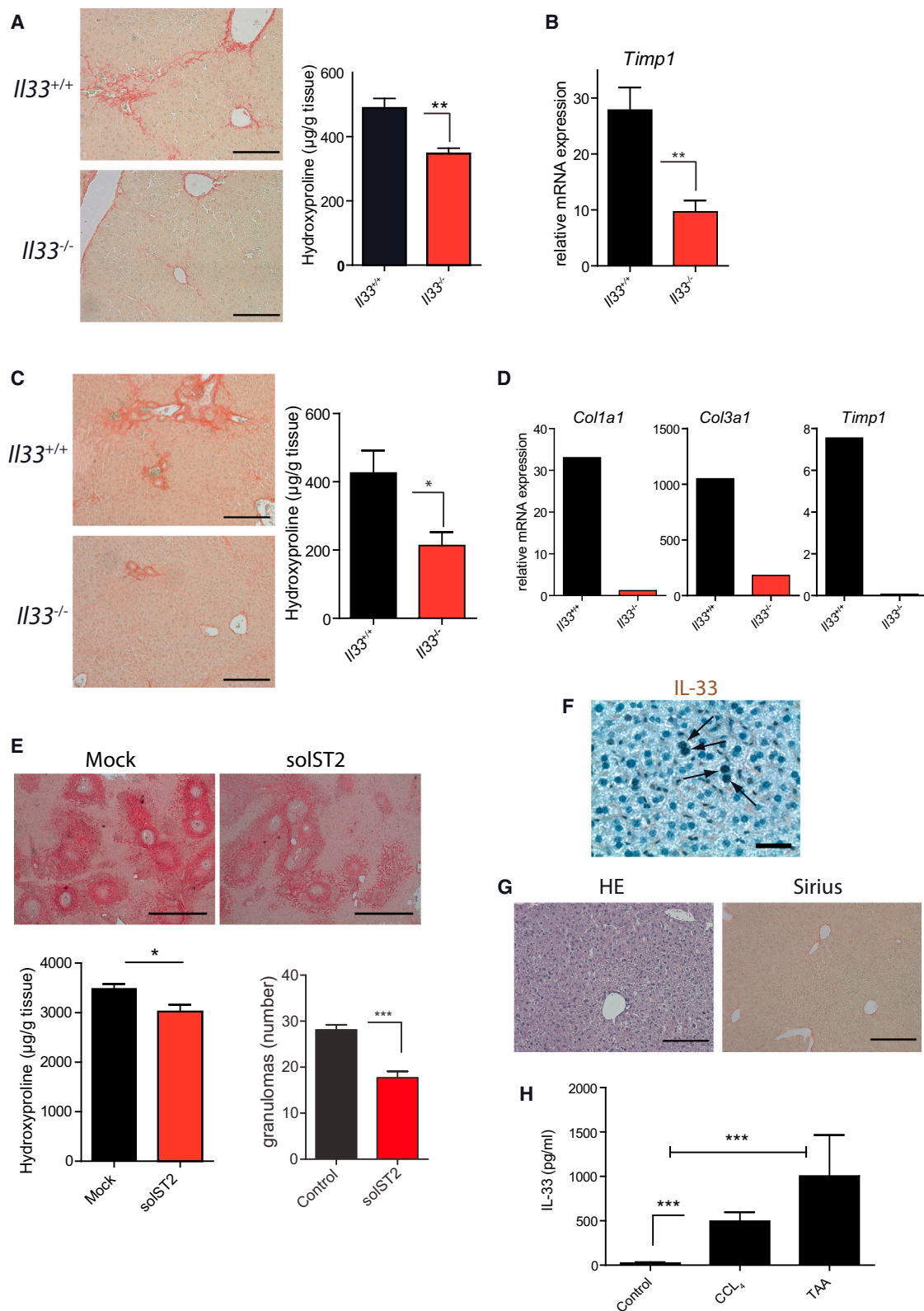


Figure 2. IL-33 Deficiency Ameliorates Experimental Fibrosis In Vivo

IL33^{-/-} or C57BL/6 control mice (n = 5 per group) were treated with CCL₄ for 4 weeks.

(A) Livers were stained for determination of fibrillar collagen depositions with Sirius Red and hydroxyproline amounts in livers were determined (*p < 0.01).

(B) *Timp1* mRNA in liver total RNA of CCL₄ treated mice or controls was quantified by qPCR (**p < 0.01).

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mice and wild-type (WT) littermates by repeated administration of CCL₄. After 4 weeks, *Il33*^{-/-} mice displayed a significant reduction in excess collagen deposits as shown by Sirius Red staining and hydroxyproline quantification (Figure 2A), whereas total hepatic collagen was similar in unchallenged *Il33*^{-/-} mice compared to controls (data not shown). Consistent with the ameliorated fibrotic phenotype, a strong reduction in mRNA expression of *Timp1*, a gene associated with HSC activation and fibrosis in humans and mice (Benyon et al., 1996; Jin et al., 2011), was observed in livers of *Il33*^{-/-} mice (Figure 2B). In addition, a biliary fibrosis model with experimental ligation of the common bile duct (BDL) was employed. Again, histologic assessment of liver tissue and total collagen quantification showed that fibrosis was markedly reduced in *Il33*^{-/-} mice compared to controls (Figure 2C). Reduction of fibrosis was further confirmed by qPCR-based quantification of fibrosis-associated gene expression with liver RNA of mice subjected to BDL for 16 days. Amounts of *Col1a1*, *Col3a1*, and *Timp1* were dramatically reduced in livers of *Il33*^{-/-} mice (Figure 2D). To evaluate whether modulation of IL-33 bioactivity affects liver pathology in Schistosomiasis, we next treated mice 3 weeks after infection with a construct for soluble ST2 (sST2), a naturally occurring IL-33 decoy receptor. Compared to controls, mice subjected to sST2 delivery developed significantly less liver disease (Figure 2E), indicating that IL-33 has proinflammatory and/or profibrotic functions in the context of parasite-induced liver disease.

Some reports describe IL-33 as intracellular protein that unfolds its ST2-dependent cytokine activity only after passive release from damaged cells (Palmer and Gabay, 2011). However, whether prominent functional roles exist for intracellular IL-33 appears to be poorly understood at this time. Therefore, one possible interpretation of our result of reduced hepatic fibrosis in *Il33*^{-/-} mice is that intracellular IL-33 possesses profibrotic properties. To address this, we delivered a vector encoding an intracellular expressed version of IL-33, including the nuclear localization signal, to *Il33*^{-/-} mice via HD. Although hepatic IL-33 protein expression was evident from IHC staining (Figure 2F), no IL-33 was detectable in the serum of those animals (data not shown) and no liver pathology was observed by histology (Figure 2G). However, when those animals were treated with CCL₄, IL-33 became detectable in the serum (Figure 2H) and hepatic fibrosis was observed (data not shown).

To obtain further information about molecular networks involved in the progression of hepatic fibrosis in this model, we next generated mRNA expression profiles of cytokines, chemokines, and profibrogenic and fibrolytic genes in livers of mice with IL-33 dependent fibrosis by qPCR. Thereby, numerous differentially regulated genes were identified (Table S1) that, when

analyzed for relationships and biological significance by Ingenuity Pathway Analysis, are clearly implicated in gene expression networks associated with development of liver fibrosis and HSC activation (Figure S2).

IL-13-Dependent Type II IL-4 Receptor Signaling Mediates IL-33-Dependent Fibrosis

Our next objective was to define pathways required for IL-33-dependent fibrotic liver disease in more detail. Because IL-13 mRNA was most prominently increased in livers of mice with IL-33 induced fibrosis and IL-33 treated *S. mansoni*-infected mice (Figures S3A and S3B), we treated *Il13*^{-/-} mice and WT littermates with mIL-33 and examined livers 4 weeks later. Sirius Red staining and quantification of hepatic collagen revealed that fibrosis in IL-33 treated *Il13*^{-/-} mice was reduced compared with controls suggesting critical profibrotic roles of IL-13 in this model (Figure 3A). IL-13 mediates biological functions via the type II IL-4 receptor, which is composed of IL-4Ra and IL-13Ra1 (Wills-Karp and Finkelman, 2008). To test whether type II IL-4R signaling is involved in IL-33 dependent fibrosis, we injected mIL-33 into *Il4Ra*^{-/-} mice. As demonstrated by Sirius Red staining and hydroxyproline assay, *Il4Ra*^{-/-} mice had a significant reduction in fibrosis compared to *Il4Ra*^{+/+} mice (Figure 3B). Furthermore, HD of an IL-13 construct, but not constructs for the Th2-related cytokines IL-5 and IL-9 (Figure S4A), clearly induced ECM depositions in livers of WT mice. In contrast, *Il4Ra*^{-/-} mice were not susceptible for IL-13 dependent fibrosis (Figure S4A). Staining of liver sections from cirrhosis patients demonstrated that both IL-4R α and IL-13R α 1 are strongly upregulated in diseased areas suggesting that these mechanistic findings in mice might translate to pathogenesis of human liver disease (Figure 3C).

IL-13-Dependent Type II IL-4R Signaling Mediates HSC Activation

Our observation that IL-13 is a dominant effector of the profibrogenic function of IL-33 prompted us to further investigate the biological role of IL-13 in hepatic fibrogenesis. Effects of IL-13 on both immune and nonhematopoietic cells have been described (Koyasu and Moro, 2011). We therefore assessed whether IL-4R α signaling in hematopoietic or nonhematopoietic cells is required for fibrogenesis by generating chimeras in which IL-4R α -deficient or WT bone marrow was transferred into recipient mice that were pretreated with clodronate-liposomes to deplete Kupffer cells. However, IL-33 induced a similar degree of hepatic fibrosis in both chimeric strains (Figure S4B). Because HSCs have been suggested to be predominant ECM-producers in the course of fibrosis, we analyzed whether IL-33-dependent IL-13 production directly regulates these nonimmune cells. We

(C and D) *Il33*^{-/-} or C57BL/6 controls underwent BDL surgery. Sixteen days later, mice were sacrificed and livers were analyzed. (C) Sections were stained with Sirius Red and hydroxyproline amounts in livers were quantified ($p < 0.05$); (D) mRNAs of selected fibrosis-associated genes in liver total RNA of BDL mice or controls were quantified by qPCR.

(E) CD1 mice were infected with ~ 30 *S. mansoni* cercaria. Three weeks later, mice were HD injected with a liver-specific expression construct for the soluble ST2 receptor. Four weeks later, mice were sacrificed. Collagen deposits in livers of mice were determined by Sirius Red staining and hydroxyproline quantification. Granulomas were counted in ten low-power fields per slide ($n = 5$ per group; *** $p < 0.001$; * $p < 0.05$).

(F–H) *Il33*^{-/-} mice were injected with 5 μ g of a liver-specific expression construct for full-length IL-33 (aa 1–260). (F) IL-33-specific immunostaining demonstrating nuclear IL-33 expression in hepatocytes. (G) Analysis of livers of mice 7 days after treatment (H) 7 days after treatment (H) 7 days after treatment with 1.8 ml/kg CCL₄ or 350 mg/kg TAA. Three days later, serum IL-33 concentrations were determined by ELISA (*** $p < 0.001$). Data are representative of at least two different experiments with similar results. Scale bars represent 200 μ m or 50 μ m (IL-33 staining). Error bars indicate SEM. See also Figure S2 and Table S1.

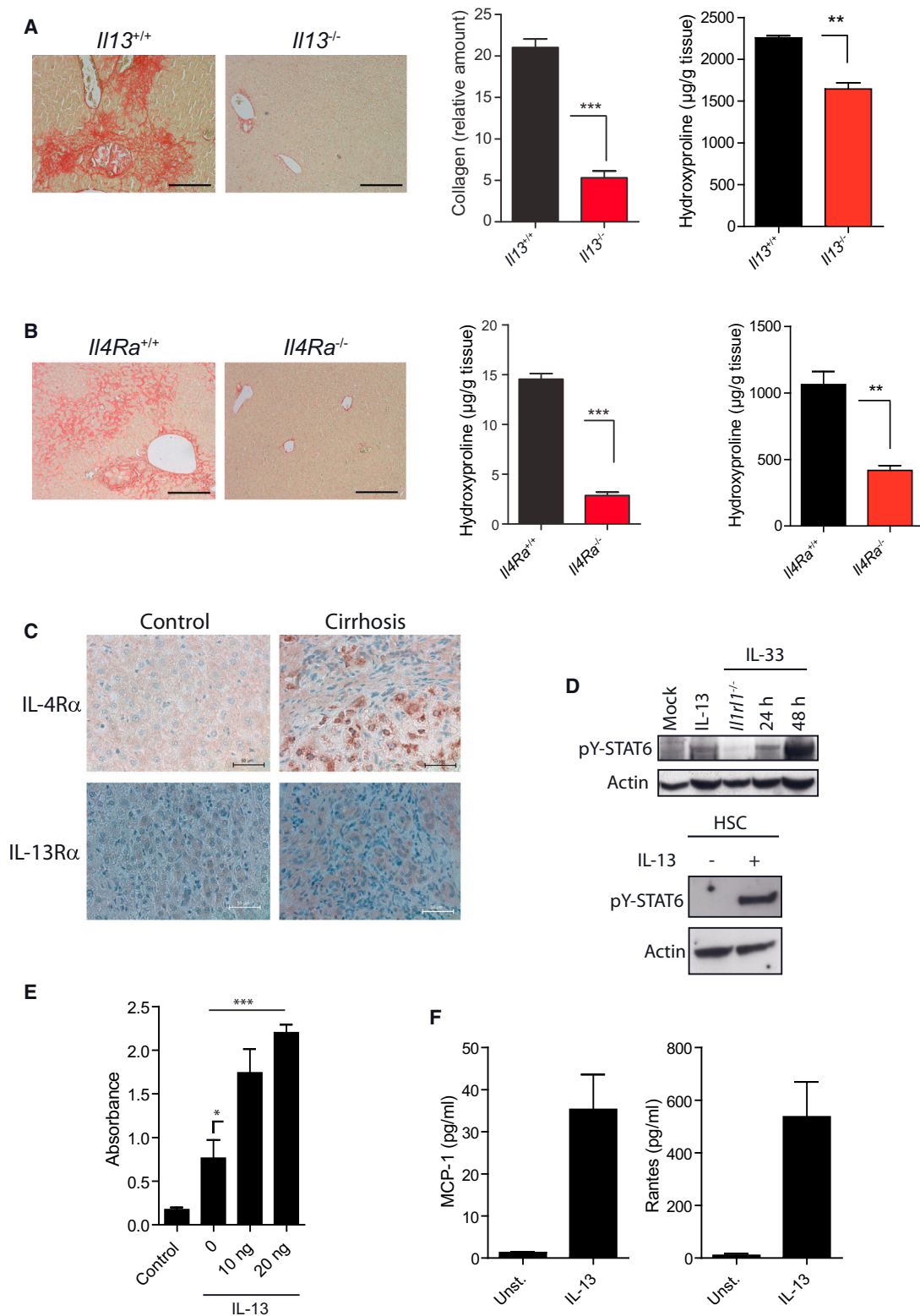


Figure 3. IL-33 Dependent Type II IL-4 Receptor Signaling Is Required for IL-33-Mediated Fibrosis and HSC Activation

IL-33 expression constructs were injected into (A) *Il13*^{-/-} mice, (B) *Il4Ra*^{-/-} mice, or Balb/c controls. Mice were sacrificed 4 weeks later and collagen depositions in livers were analyzed by Sirius Red staining or hydroxyproline assay (n = 10 mice per group; ***p < 0.001; **p < 0.01). Scale bars represent 200 μm.

(C) Representative pictures of liver tissue sections from healthy controls or patients with liver cirrhosis that were stained for IL-4Rα or IL-13Rα by immunohistochemistry. Scale bars represent 50 μm.

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isolated HSC and found that they produced mRNAs for both components of the functional type II IL-4R, IL-4R α , and IL-13R α 1. In addition, surface expression of IL-13R α 1 was demonstrated by flow cytometry (Figure S4C). Immunoblot analysis with liver lysates demonstrated increased STAT6 phosphorylation after injection of mIL-33 in vivo (Figure 3D, top panel). Moreover, purified primary HSC, stimulated with IL-13 ex vivo, responded by phosphorylation of STAT6, a critical transcription factor for IL-4- and IL-13-dependent cellular responses (Hebenstreit et al., 2006) (Figure 3D, lower panel). Additionally, stimulation of HSC with IL-13 increased their proliferation in vitro (Figure 3E) and elicited an IL-13-dependent increase in mRNA of profibrotic genes (Figure S4D) and chemokines (Figure 3F). Collectively, these data suggest that IL-33 through IL-13 modulates HSC functions.

ILC2 Are Increased in Livers of Mice with Hepatic Fibrosis

Different cell types have been described as possible sources of IL-13 in response to IL-33 stimulation (Besnard et al., 2011; Kroeger et al., 2009; Stolarski et al., 2010). To identify which cells are the dominant producers of IL-33-dependent IL-13 in this system, we first assessed whether immune cells or liver-resident cells are critical targets of IL-33 by generating chimeras in which ST2-deficient or WT BM cells were transferred into WT recipients (Figure 4A). Interestingly, mIL-33 treatment induced different responses in the two strains: whereas mice reconstituted with WT BM developed severe fibrotic disease, chimeras with defective IL-33 signaling in BM-derived cells did not show liver pathology or excess collagen (Figures 4B and 4C).

To characterize the role of these IL-33-responsive hematopoietic cells further, we determined the contribution of lymphocytes for progression of liver fibrosis. *Rag1*^{-/-} mice treated with mIL-33 developed marked liver pathology at 4 weeks after HD (Figure 4D). In fact, Sirius Red staining and hydroxyproline determination indicated that the extent of fibrosis was more pronounced in these mice compared to controls (Figures 4D and 4E). In contrast, *Rag1*^{-/-}*gc*^{-/-} mice, which—in addition to B and T cells—lack γ C-dependent lymphocytes, e.g., natural killer cells and ILC, were resistant to IL-33 mediated hepatic fibrosis (data not shown). We next did qPCR analysis to compare the expression of several fibrosis-associated genes in livers from mIL-33-treated *Rag1*^{-/-} and WT mice. Indeed, and consistent with our data described above, hepatic mRNAs for Th2 cell-associated cytokines, particularly IL-13, were strongly elevated in *Rag1*^{-/-} mice. In contrast, amounts of the antifibrotic cytokine interferon- γ were lower in *Rag1*^{-/-} mice (Figure 4F).

Recently, several reports have described innate lymphoid cell populations (nuocytes, natural helper cells, innate helper cells, ILC2) that respond to stimulation with IL-25 and/or IL-33, are capable of producing substantial amounts of IL-13 and are important for host responses to infections (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010b). Our observation that IL-33 induced hepatic fibrosis developed inde-

pendently of adaptive immune cells and largely depended on IL-13, but not on the presence of basophils and mast cells (Figures S5A and S5B), prompted us to analyze the functional role of ILC2 in fibrotic liver disease. First, we treated *Il4-eGFP* (4get) mice, a reporter strain marking type-2 competent cells by expression of eGFP (Mohrs et al., 2001), with mIL-33 and analyzed eGFP expression in isolated liver cells by flow cytometry (Figure 5A). As a result, the number of eGFP⁺Sca-1⁺ cells increased dramatically with IL-33 treatment. Sca-1 is expressed on hematopoietic progenitors and was previously shown to be expressed by ILC2 (Saenz et al., 2010a). In further experiments, we were able to recover small numbers of lin⁻, CD45⁺ cells, characterized by expression of IL-33R, IL-7R α , CD44, CD90.2, ICOS, and Sca-1, resembling ILC2, from the livers of naive mice. In livers of mIL-33-treated mice, we found these cells strongly enriched (Figure 5B), and moreover, these ILC2 produced IL-13 as shown by intracellular flow cytometry analysis (Figure 5C). Recently, the transcription factor ROR α has been shown to be critical for nuocyte development (Wong et al., 2012), and mice treated with mIL-33 had increased liver ROR α mRNA expression compared to controls (Figure 5D). To determine a potential impact of ROR α on the development of liver ILC2, we analyzed ROR α expression in purified liver ILC2. Quantitative analysis of ROR α mRNA showed high ROR α abundance in ILC2 compared to ROR- γ t, a factor involved in the developmental program of other ILC lineages (ILC3) (Figure 5E). In addition, hepatic numbers of lin⁻Sca-1⁺ICOS⁺ cells were reduced in chimeric mice reconstituted with BM from mice lacking ROR α , suggesting that liver ILC2 are developmentally related to ILC2 described in other tissues (Wong et al., 2012) (Figure 5F). Next we investigated the frequency of ILC2 in mice with hepatic fibrosis. We found that lin⁻ST2⁺ICOS⁺ cells were increased in livers of mice with CCL₄ induced fibrosis (Figure 5G). However, in livers of CCL₄ treated *Il1rl1*^{-/-} mice, ILC2 amounts were lower than in WT mice (Figure 5H). Moreover, hepatic ILC2 numbers were also increased in *S. mansoni*-infected mice (Figure 5I), whereas blockade of IL-33 by sST2 treatment reduced liver ILC2 and ROR α mRNA (Figure 5J). These findings indicate that IL-33 signaling is important for hepatic accumulation of ILC2 during chronic liver disease. Similar numbers of hepatic ILC2 were present in naive *Il33*^{-/-}/4get versus *Il33*^{+/+}/4get mice, suggesting that IL-33 signaling might not be required for ILC2 survival in conditions of liver homeostasis (Figure S5C). Accordingly, hepatic overexpression of IL-25 expanded liver ILC2 in the absence of both IL-33 and ST2 (Figures S5D and S5E). However, although hepatic IL-25 mRNA was somewhat increased in mice with CCL₄ or infection-dependent liver fibrosis (Figure S5F), no IL-25 protein could be detected in the sera of these mice, suggesting that IL-33 rather than IL-25 is responsible for ILC2 expansion in chronic liver diseases.

ILC2 Mediate Hepatic Fibrosis

IL-33-treated *Rag1*^{-/-} mice showed higher numbers of ILC2 in their livers than WT mice (data not shown), consistent with a

(D) Detection of activated STAT6 by immunoblot in whole liver lysates (top panel) or IL-13 stimulated HSC (lower panel).

(E) HSCs were stimulated in vitro with rIL-13 for 4 days. Cell growth was assessed with WST-1 assay. Control: medium alone (**p < 0.001; *p < 0.05).

(F) HSCs were cultivated for 48 hr with or without the presence of 30 ng/ml IL-13. Chemokines in supernatants were quantified by ELISA. Data are representative of at least two different experiments. Error bars indicate SEM. See also Figure S3.

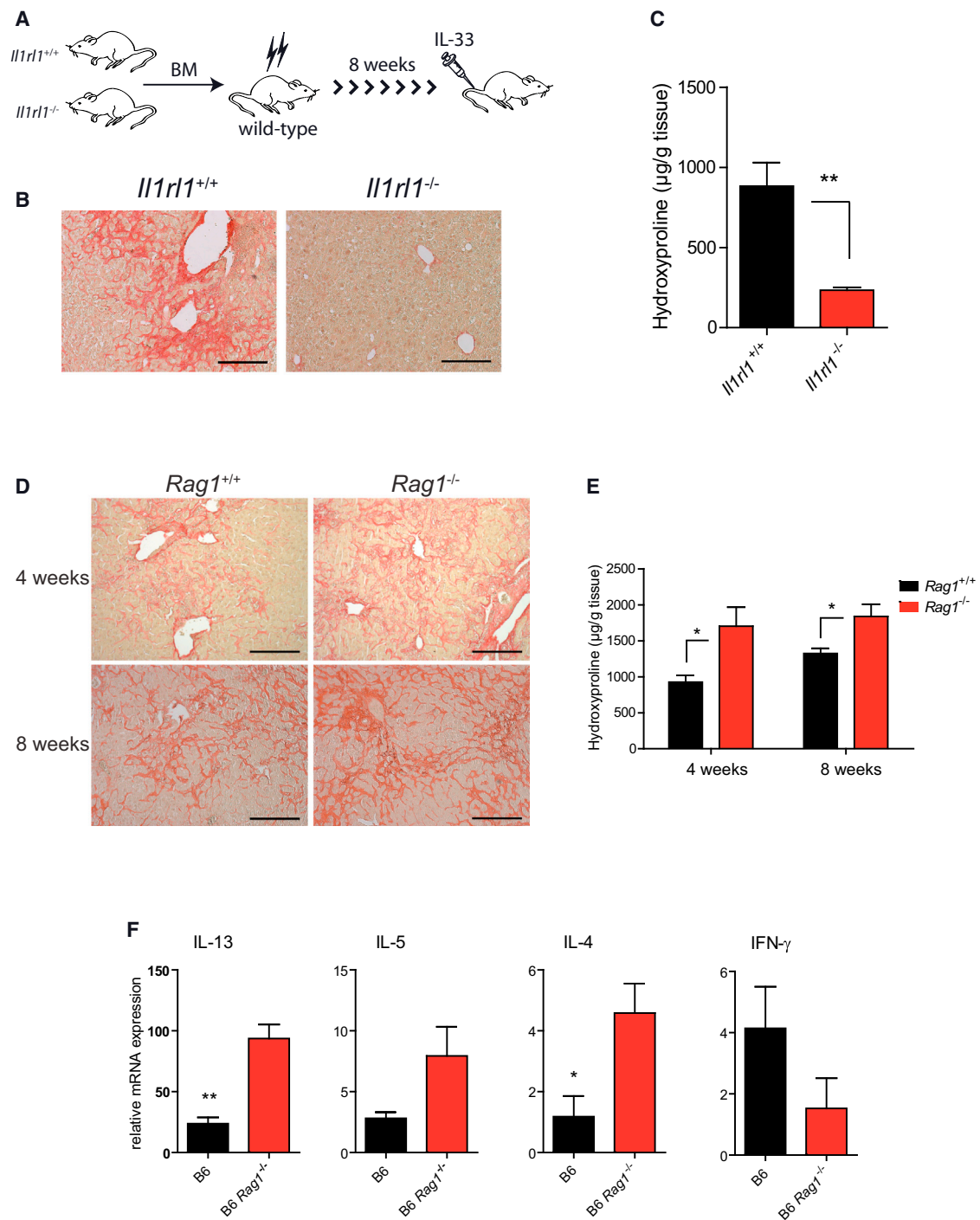


Figure 4. Innate Immune Cells Promote IL-33-Dependent Fibrosis

(A) *Il1rl1*^{-/-} or *Il1rl1*^{+/+} BM cells were i.v. injected into irradiated C57BL/6 recipient mice. Eight weeks later, mice received mIL-33 via HD.

(B) Representative Sirius Red stainings of livers of mice 4 weeks after treatment.

(C) Hepatic collagen content was determined by hydroxyproline quantification (n = 5 per group; **p < 0.01).

(D–F) IL-33 expression constructs were injected into C57BL/6 or *Rag1*^{-/-} mice. (D) Sirius Red stainings of livers of mice 4 or 8 weeks after treatment. (E) Total collagen in livers was determined by hydroxyproline quantification (n = 5–6 mice per group; *p < 0.05). (F) Hepatic mRNAs of fibrosis-associated genes 4 weeks after mIL-33 treatment in mice were quantified by qPCR (n = 5 per group; **p < 0.01; *p < 0.05). Data are representative of at least two different experiments with similar results. Scale bars represent 200 μm. Error bars indicate SEM. See also Figure S4.

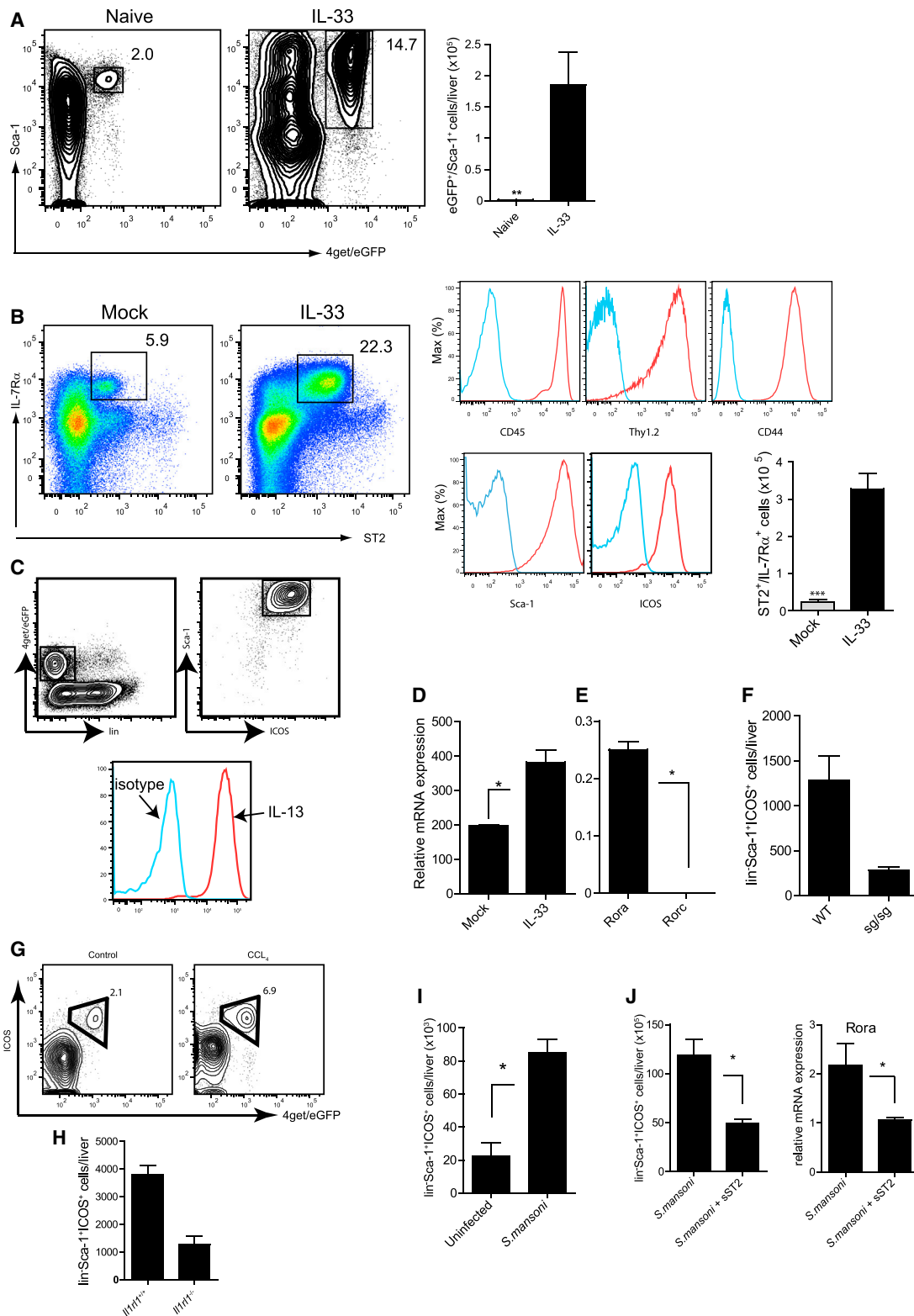


Figure 5. IL-33 Expands Liver Resident Type-2 Related ILC that Produce IL-13

(A) Flow cytometric analysis of isolated hepatic immune cell populations from naive IL-4/eGFP reporter mice or 5 days after treatment with mIL-33 ($n = 3-4$ per group; $**p < 0.01$).

(B) Hepatic immune cell populations of mock or mIL-33 injected *Rag1*^{-/-} mice were isolated and depleted from lin⁺ (CD5⁺, B220⁺, CD11b⁺, Gr1⁺, 7/4⁺, Ter119⁺) cells. The expression of IL-7R α and ST2 was analyzed by flow cytometry. Gated IL-7R α ⁺/ST2⁺ cells were further analyzed for expression of CD45, Thy1.2, CD44, (legend continued on next page)

more severe IL-33-dependent fibrotic phenotype (Figure 4D). To directly investigate a possible link between ILC2 and liver tissue remodeling, we performed depletion and ILC2 transfer experiments. ILC2 counts can be minimized in livers of *Rag1*^{-/-} mice by treatment with α Thy1.2 (CD90.2) antibodies (Figure 6A). ILC2 depletion prior to IL-33 administration strongly reduced IL-33-dependent hepatic fibrosis (Figure 6B), although α Thy1.2 treatment 2 weeks after mIL-33 administration was less protective (Figure S6A). Moreover, α Thy1.2 treatment of *Rag1*^{-/-} mice (Figure S6B; Figure 6C) and C57BL/6 WT mice (Figure 6D) resulted in significantly decreased CCL₄-dependent fibrosis as evident by reduced Sirius Red staining and hydroxyproline assay at week 4. Similarly, ILC2 depletion in chronic CCL₄-dependent fibrosis was also efficient in *Rag1*^{-/-} mice reconstituted with T and B cells from Thy1.1⁺ mice (Figure 6E) and resulted in decrease expression of the fibrosis-associated genes *Acta1* and *Timp1* (Figure 6F). Next, we adoptively transferred purified ILC2 from *Il1rl1*^{+/+} mice into *Il1rl1*^{-/-} mice and treated these mice with mIL-33. Analysis of liver tissue 4 weeks after treatment showed that ILC2 treated mice but not controls had marked inflammatory cell infiltrations and collagen depositions. In contrast, no such liver alterations were observed in mIL-33 treated *Il1rl1*^{-/-} control mice that received no ILC2 (Figure 7A). Similarly, ILC2 transfer into *Il1rl1*^{-/-} mice subjected to the TAA model to mimic chronic hepatocellular stress clearly increased the degree of hepatic fibrosis (Figure 7B). Moreover, in a model of hepatic granuloma formation by intraportal vein transfer of Schistosoma eggs (Figure S7C) the size of egg induced granulomas in *Il1rl1*^{-/-} mice was significantly increased when ILC2 were adoptively transferred (Figure 7C).

Given that *Il13*^{-/-} mice are largely protected from IL-33-dependent fibrosis (Figure 3A) and that ILC2-transferred *Il1rl1*^{-/-} mice treated with a vector encoding a neutralizing IL-13R α 2-Fc fusion protein developed less IL-33-dependent fibrosis (Figure S7A), we directly tested whether ILC2 promote hepatic tissue remodeling via IL-13 production. By transferring WT ILC2 into *Il13*^{-/-} mice, we demonstrated that ILC2 even when they are the only IL-13 secreting cell-type were sufficient to mediate fibrosis (Figure 7D). Accordingly other ST2⁺ IL-13 producing lineages such as basophils and mast cells were less important for IL-33 mediated disease (Figure S5 A and S5B) and moreover other cytokines that were reported to be produced by ILC2 such as IL-9 (Wilhelm et al., 2011) are not as critical for IL-33 dependent fibrosis as IL-13 (Figure S7B). Collectively, these data suggest a critical role of IL-33 dependent ILC2 in promoting

hepatic fibrosis. Results from these experiments indicate that IL-33 is critical for hepatic accumulation of ILC2 and that ILC2, through an IL-13-dependent mechanism, are both required and sufficient for hepatic fibrosis.

DISCUSSION

Hepatic fibrosis develops as a consequence of chronic liver infections, such as HBV, HCV, or Schistosomiasis, or can occur as a result of sustained metabolic or biliary imbalances. Irrespective of the underlying cause, hepatic fibrosis in humans and experimental animal models is closely associated with persistent activation of inflammatory pathways. However, early molecular and cellular networks that link hepatocellular stress to inflammation and subsequently to initiation and progression of liver fibrosis remain poorly defined. Here we present compelling evidence that IL-33—released from liver cells in the context of hepatocellular stress—is a key mediator of hepatic fibrosis in vivo. We show that IL-33 release from liver cells leads to activation and expansion of IL-13 producing liver-resident ILC2 and identify a previously unrecognized role for these cells during hepatic tissue remodeling.

We demonstrate that elevated concentrations of IL-33 protein in the serum and liver tissue relate to the condition of hepatic fibrosis in humans and also are observed across different mouse models of hepatic fibrosis, which is consistent with a recent study reporting an association of IL-33 overexpression with hepatic fibrosis (Marvie et al., 2010). Importantly, our data provide multiple lines of evidence that intrahepatic release of IL-33 in the context of stress-dependent cellular damage is associated with fibrogenic changes in the liver in vivo. Taken together, these results provide a rationale for investigation of serum IL-33 as possible noninvasive diagnostic biomarkers of chronic hepatocellular injury and fibrosis; such biomarkers might be valuable in uncovering early inflammatory and fibrogenic events and might complement existing clinical markers of hepatic fibrosis.

To address the question of whether IL-33 was not only sufficient but also required for hepatic fibrosis, IL-33-deficient mice were challenged in two different models of hepatic fibrosis: CCL₄ and BDL. In both models, IL-33-deficient animals developed significantly reduced collagen deposits compared to WT littermates. Similar to the reduction in collagen, expression of ECM-associated genes was diminished in IL-33-deficient mice. Collectively, these data support the concept that IL-33 contributes to severe hepatic fibrosis in vivo.

ICOS, and Sca-1. Blue histograms represent isotype controls. Numbers of lin⁻ IL-7R α ⁺/ST2⁺ cells in livers of controls or mIL-33 injected *Rag1*^{-/-} mice (n = 4–8 per group; ***p < 0.001).

(C) Hepatic immune cells from 4get/*Rag1*^{-/-} mice were stimulated for 4 hr with PMA and Ionomycin. Surface expression of Sca-1 and ICOS and intracellular IL-13 expression were analyzed by flow cytometry.

(D) Total liver RNA from 5 days control or mIL-33 treated mice was isolated and used for ROR α -specific qPCR analysis.

(E) Total RNA from sort-purified liver ILC2 isolated from *Rag1*^{-/-} mice treated for 5 days with mIL-33 was used for ROR α - and ROR γ -specific qPCR (*p < 0.05).

(F) Flow-cytometry-based quantification of lin⁻ Sca-1⁺ICOS⁺ cells in irradiated mice reconstituted with BM from WT or sg/sf mice (n = 2–4 per group).

(G) Flow cytometry analysis of lin⁻, eGFP⁺, ICOS⁺ cells in livers of 4get mice treated for 4 weeks with CCL₄.

(H) *Il1rl1*^{+/+} WT or *Il1rl1*^{-/-} mice were treated for 4 weeks with CCL₄ before hepatic immune cells were isolated and analyzed by flow cytometry for the presence of lin⁻ Sca-1⁺ICOS⁺ cells. Data are representative of at least two different experiments with five mice per group with similar results.

(I) CD1 mice were infected with ~200 *S. mansoni* cercaria or left uninfected. Three weeks later, mice liver ILC2 numbers were determined by flow cytometry.

(J) CD1 mice were infected with ~30 *S. mansoni* cercaria. Four weeks later, they were HD injected with an expression construct for sST2. Four weeks later, mice were sacrificed and liver ILC2 numbers were determined. Hepatic Rora mRNA was quantified by qPCR (n = 5 per group; *p < 0.05). Error bars indicate SEM. See also Figure S5.

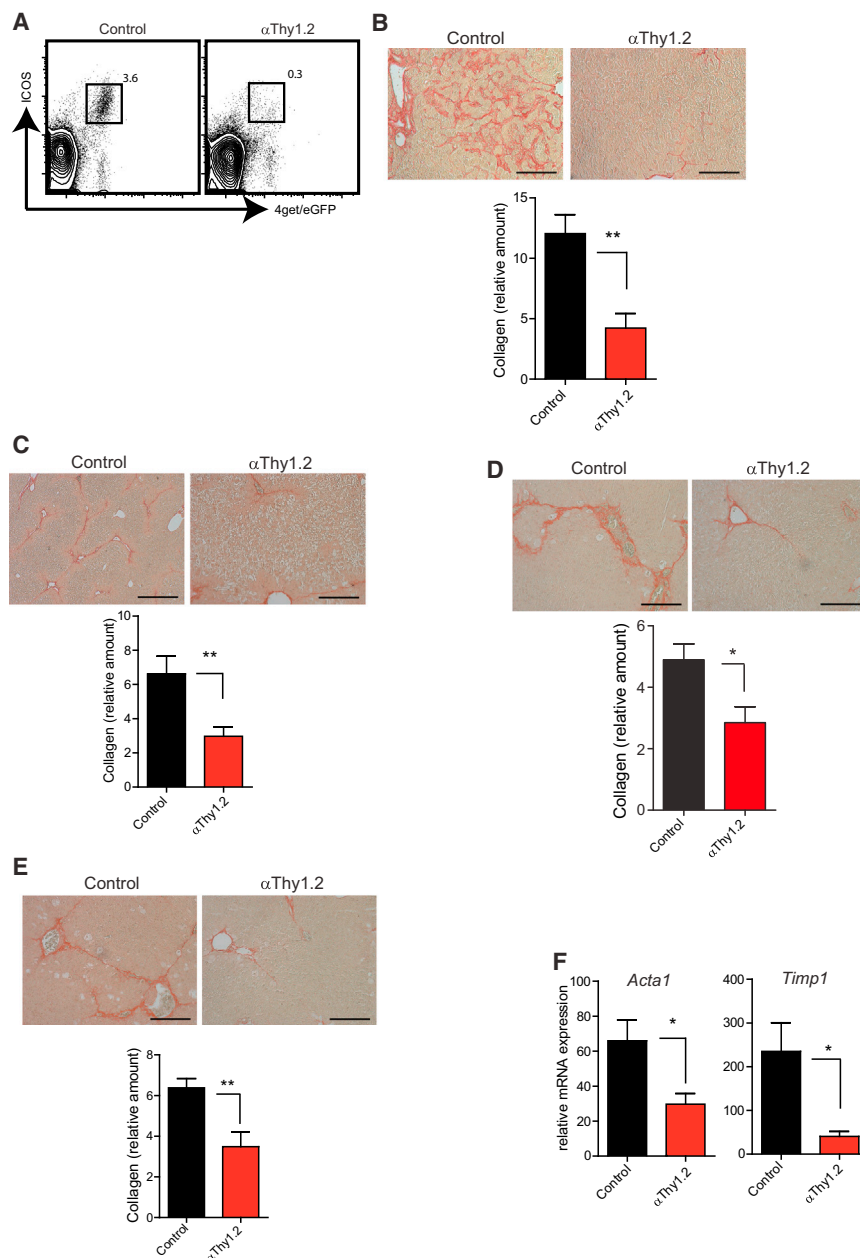


Figure 6. ILC2 Are Important Mediators of Hepatic Fibrosis

(A) Naive 4get/*Rag1*^{-/-} mice were treated every other day with 200 μ g α Thy1.2 mAb for 6 days. *lin*⁻eGFP⁺ICOS⁺ cells in livers were analyzed by flow cytometry.

(B) 4get/*Rag1*^{-/-} mice were treated every 3 days with 200 μ g α Thy1.2 mAb for 4 weeks. At day 6, mice were HD injected with mCL-33. Collagen depositions in livers of mice were determined by Sirius Red staining (n = 5 per group; **p < 0.01).

(C and D) *Rag1*^{-/-} (C) or C57BL/6 (D) mice were treated every 3 days with 200 μ g α Thy1.2 or control mAbs for 4 weeks. Starting at day 6, mice were treated twice weekly with 0.8 ml/kg CCL₄ for 4 weeks. Collagen deposits in livers of mice were determined by Sirius Red staining (n = 5 per group; **p < 0.01). Scale bars represent 200 μ m.

(E and F) *Rag1*^{-/-} mice were intravenously reconstituted with 4×10^6 purified splenic T and B cells from Thy1.1⁺ mice. Seven days later, mice were treated every 3 days with 200 μ g α Thy1.2 mAb for 4 weeks. Starting at day 6, mice were treated twice weekly with 0.8 ml/kg CCL₄. Collagen deposits in livers of mice were determined by Sirius Red staining. Scale bars represent 200 μ m. Hepatic mRNAs of *Acta1* and *Timp1* were determined by qPCR (n = 6–7 per group; **p < 0.01; *p < 0.05). Data represent two independent experiments with similar results. Error bars indicate SEM. See also Figure S6.

Transcriptional profiling identified IL-13, a cytokine previously described as inflammatory and fibrogenic mediator in other organs, as one of the most prominently upregulated genes during IL-33-mediated hepatic fibrosis. Indeed, *Il13*^{-/-} and *Il4Ra*^{-/-} mice were markedly protected from IL-33-mediated disease suggesting that IL-33 and IL-13 constitute a profibrotic axis in the course of the disease. This concept is further supported by our studies demonstrating IL-33- or IL-13-dependent hepatic activation of STAT6 in vivo and direct activation of signaling, proliferation, and gene expression of HSC in vitro. A recent report suggested that IL-13 activates STAT6 but also SMAD-family transcription factors in rat HSC. Through this mechanism, IL-13 bypasses requirements for profibrotic transforming growth factor- β (TGF- β) signaling for full HSC activation (Liu et al.,

2011). Consistent with the murine data, we have demonstrated elevated hepatic protein expression of both components of the functional IL-13 receptor, IL-4R α and IL-13R α , in human liver cirrhosis sections compared to controls. This indicates increased sensitization to IL-13-dependent signals in human fibrotic liver disease and suggests at least partial functional congruence of profibrotic hepatic networks in mouse and human. Taken together, these findings suggest a

hepatic IL-33/IL-13 axis, which promotes HSC activation and triggers a potent fibrogenic response.

A number of different cell types have been identified as candidates for IL-13 production after IL-33 stimulation (Liew et al., 2010). Although other IL-13-producing cell types such as lymphocytes, basophils, and mast cells did not contribute profoundly, we identified in a series of in vivo experiments a γ c-dependent non-T and non-B cell lymphoid cell population that was a strong IL-13 producer and important pathogenic cell type in our system. These cells depend on the expression of the transcription factor ROR α and express a panel of cell surface receptors characteristic of ILC2, a recently described innate lymphocyte subset with a key role in host defense to viral or parasitic infection (Chang et al., 2011; Kang et al., 2012; Moro

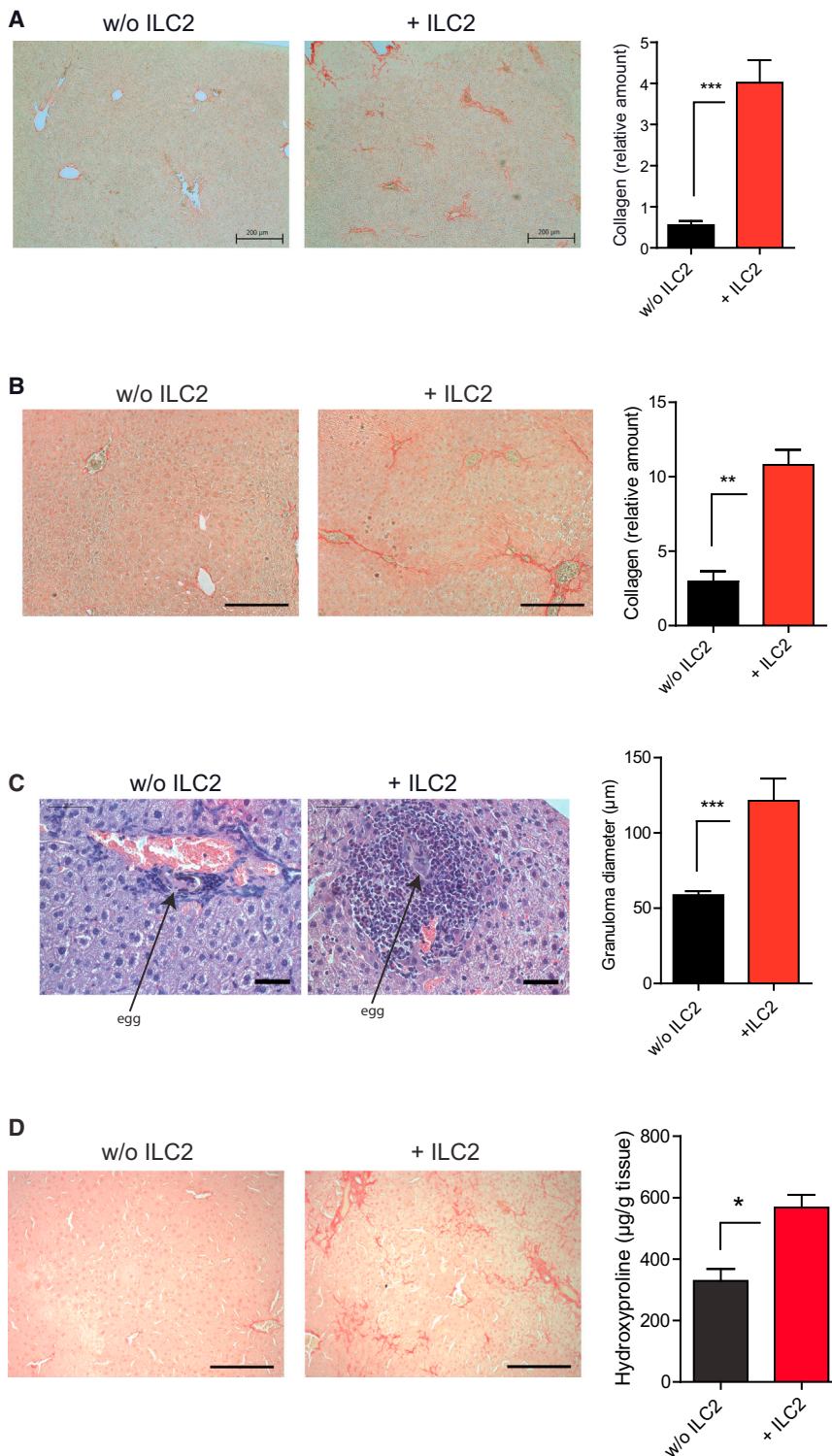


Figure 7. Adoptive Transfer of ILC2 Restores Hepatic Fibrosis

(A) *Il1rl1*^{-/-} mice were injected i.v. with 2×10^6 sorted, WT ILC2, or saline only. Twenty-four hr later, mice were treated with mIL-33. Hepatic collagen content was assessed by Sirius Red staining ($n = 4$ per group; *** $p < 0.001$).

(B) *Il1rl1*^{-/-} mice were injected i.v. with 2×10^6 ILC2 or saline. Subsequently, mice were treated i.p. for 4 weeks with TAA as described in methods. Hepatic collagen content was assessed by Sirius Red staining ($n = 5$ per group; $p < 0.01$).

(C) *Il1rl1*^{-/-} mice were injected i.v. with 2×10^6 ILC2. Twenty-four hr later, mice were injected with 5,000 freshly isolated *S. mansoni* eggs via the portal vein system. Four weeks later, mice were sacrificed and granuloma formation was analyzed in HE stained liver sections ($n = 3-4$ per group; $p < 0.001$).

(D) *Il13*^{-/-} mice were injected i.v. with 2×10^6 ILC2 from WT Balb/c mice. Twenty-four hr later, mice were treated with mIL-33 via HD. Collagen depositions in livers were determined by Sirius Red staining and hydroxyproline assay ($n = 4$ per group; ** $p < 0.01$). Scale bars represent 200 μ m (A, B, D) or 50 μ m (C). Data are representative of at least two independent experiments with similar results. Error bars indicate SEM. See also Figure S7.

mice restored this strains' susceptibility to fibrosis in the different models that we employed indicating that intrahepatic ILC2 have a critical role in promoting IL-33-mediated liver fibrosis in vivo. For the first time to our knowledge this report describes a role for ILC2 outside the realm of host defense at mucosal surfaces and establishes a role of ILC2 in hepatic tissue remodeling networks via IL-33-dependent IL-13 production. Further studies are required to clarify the exact role of both IL-33 and ILC2 in normal liver physiology, although our results indicate that IL-33 might not be essential for survival of hepatic ILC2 in conditions of normal liver function. Recently, IL-33 upregulation in hepatocytes and a protective role of IL-33/ST2 signaling in ConA hepatitis, a model of acute, fulminant hepatitis in humans, has been described (Arshad et al., 2011; Volarevic et al., 2012). Although in another study *Il33*^{-/-} mice do not show differential susceptibility to ConA hepatitis (Oboki et al., 2010), it is tempting to speculate that IL-33 might exert a protective role in tissue repair responses following transient injury, whereas sustained IL-33 release due to chronic hepatocellular stress drives pathological tissue remodeling in the liver, as demonstrated here. Consistent with this idea, IL-33-dependent ILC2 promote early lung tissue homeostasis

et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010b). Reduction of ILC2 numbers by administration of a depleting mAb to Thy1.2 correlated with reduced sensitivity to hepatic fibrosis after HD administration of IL-33, as well as after chronic CCL₄ challenge. Moreover, adoptive transfer of ILC2 purified from livers of WT mice into IL-33 unresponsive *Il1rl1*^{-/-}

tis (Oboki et al., 2010), it is tempting to speculate that IL-33 might exert a protective role in tissue repair responses following transient injury, whereas sustained IL-33 release due to chronic hepatocellular stress drives pathological tissue remodeling in the liver, as demonstrated here. Consistent with this idea, IL-33-dependent ILC2 promote early lung tissue homeostasis

during acute influenza infection (Monticelli et al., 2011) but also mediated severe airway hyperreactivity and asthma (Barlow et al., 2012; Bartemes et al., 2011; Chang et al., 2011). Thus, ILC2 might have evolved to establish a direct link between tissue damage responses and host defense, and some of the stereotypical Th2-associated genes might more accurately be viewed as dual role factors coordinating host defenses and tissue remodeling.

Lastly, our findings validate the concept of therapeutic modulation of IL-33 or IL-33-dependent ILC2 responses for treatment of conditions that involve chronic hepatic inflammation and fibrosis.

EXPERIMENTAL PROCEDURES

Animals and Mouse Models

C57BL/6, Balb/cJ, *Rag1*^{-/-}, sg/sf, and *IL4Rα*^{-/-} (Balb/c background) mice were obtained from Jackson Laboratory. *IL13*^{-/-} mice were kindly provided by A. McKenzie. 4get/eGFP reporter (Mohrs et al., 2001) and Mcpt8Cre (Ohnmacht et al., 2010) mice were obtained from D. Voehringer. *IL33*^{-/-} mice were recently described (Louten et al., 2011). *IL11r1*^{-/-} mice were originally obtained from Shizuo Akira's laboratory and backcrossed to the C57BL/6 background for ten generations. We used 8- to 12-week-old age- and sex-matched mice for experimental procedures. Chronic CCL₄-dependent hepatic fibrosis was induced by eight intraperitoneal injections of CCL₄ (Merck) at 0.8 ml/kg in mineral oil (Sigma). For eliciting thioacetamide (TAA), induced toxic fibrosis TAA (Sigma) was intraperitoneally (i.p.) injected at 200 mg/kg thrice a week for 4 weeks. In some experiments, cholestasis-dependent fibrosis was induced by surgical ligation of the common bile duct under ketamine and xylazine anesthesia. Sham-operated mice underwent laparotomy without ligation. For analysis of hepatic IL-33 expression after *Schistosoma* infection, mice were infected with ~176–200 (high dose) or ~30 (low dose) *S. mansoni* cercaria larvae (Blank et al., 2010).

For generation of bone marrow chimeras, 2 × 10⁷ cells from femurs and tibias of donor mice were intravenously (i.v.) injected into lethally irradiated (10Gy: C57BL/6; 7.8 Gy: Balb/c) recipient mice. In some experiments, recipients were i.p. pretreated for 24 hr with 200 μl clodronate liposomes to deplete Kupffer cells (van Rooijen and van Kesteren-Hendrikx, 2003). Mice were let to recover for 8 weeks followed by mIL-33 injection into the tail veins. Animal experiments were approved by the governments of Rheinland-Pfalz and Mittelfranken.

Liver Cell Isolation Procedures

Livers were aseptically removed from experimental mice, and nonparenchymal liver cells were isolated with the gentleMACS device according to the manufacturer's instructions (Miltenyi Biotec). Briefly, livers were digested two times for 30 min in Krebs-Ringer solution containing 5,000 U Collagenase IV (Sigma) and 30,000 U/ml DNase I (Roche) in gentleMACS C tubes at 37°C. Subsequently, cells were passed through a 100 μm filter mesh and remaining hepatocytes were removed by centrifugation at 20 g for 4 min.

For further purification of HSCs, pellets of nonparenchymal liver cells suspended in 15% (w/v) iodixanol were overlaid with a solution containing 10% (w/v) iodixanol and HBSS and centrifuged at 1,400 g for 20 min as described previously (Elsharkawy et al., 2010). HSCs were collected at the interface of the low-density barrier. Immediately after cell isolation, purity of isolated HSC was examined by analysis of autofluorescence excitation under UV light or by FACS for autofluorescence and negativity for CD45.

For further purification of immune cell populations, cells were suspended in 30% Percoll, underlaid with 100% Percoll, and centrifuged at 1,400 g for 20 min. Cells from the interface were collected, counted, and processed for FACS analysis or cell culture. In some experiments, ILC2 populations were enriched with a lineage cell depletion kit according to the manufacturer's instructions (Miltenyi Biotec).

Flow Cytometry

Single-cell suspensions from livers were stained with combinations of the following fluorochrome-tagged antibodies (all from eBioscience unless

specified otherwise): Allophycocyanin-conjugated anti-CD45 (30-F11), anti-Sca-1 (D7), anti-Thy1.2 (53-2.1), anti-CD44 (IM7), Fluoresceinisothiocyanat-conjugated anti-T1-ST2 (DJ8, MD Biosciences, Zuerich, Switzerland), Phycoerythrin-conjugated anti-IL-13Rα1 (13MOKA), anti-IL-13 (eBio13A), anti-ICOS (7E.17G9), and Brilliant Violet 421-conjugated anti IL-7Rα (A7R34), anti-mouse lineage-cocktail (Biolegend). Samples were measured on an LSRFortessa cell analyzer (BD Biosciences) and were analyzed with Flowjo (Treestar).

In Vivo Depletion of ILC2

For depletion of ILC2 in mice anti-Thy1.2 monoclonal Ab (clone 30H12) from BioXCell was used. We administered 200 μg per mouse i.p. every 3 days as described in the figure legends.

Adoptive Transfers of Liver ILC2

Single-cell suspensions from livers of 4get/*Rag1*^{-/-} mice treated with mIL-33 for 5 days were enriched for lineage cells using a lineage cell depletion kit according to the manufacturer's instructions (Miltenyi Biotec). Cells were stained with PE-conjugated antibodies to ICOS- and APC-conjugated antibodies to Sca-1 and 4get/eGFP, and PE- and APC-positive cells were collected with a FACSaria II cell sorter (Becton Dickinson). Sorted cells were adoptively transferred to recipient mice by i.v. tail vein injection.

Cytokine and Chemokine Measurements

For determination of mouse and human IL-33, soluble ST2 ELISA and IL-13-specific Duoset ELISA Kits from R&D Systems were used. Chemokine concentrations in cell culture supernatants and organ lysates were measured by a mouse Flowcytometry kit (Ebioscience) according to manufacturer's instructions. All studies with human material were approved by the ethics committee of the University Hospital of Erlangen.

Cell Proliferation Assay

Isolated HSCs were stimulated with 20 ng/ml rIL-13 (R&D Systems, Wiesbaden, Germany) for 72 hr, WST-1 proliferation reagent (Roche) was added (1:10 dilution), followed by incubation for 4 hr at 37°C. The absorbance was measured at 450 nm.

Statistical Analysis

Student's *t* tests or Mann-Whitney tests were performed with Graphpad 5 software (Prism). *p* values < 0.05 with a 95% confidence interval were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2013.07.018>.

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