Type 2 immunity is protective in metabolic disease but exacerbates NAFLD collaboratively with TGF-β

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Nonalcoholic fatty liver disease (NAFLD) is the most common progressive liver disease in developed countries and is the second leading indication for liver transplantation due to the extensive fibrosis it causes. NAFLD progression is thought to be tied to chronic low-level type 1 inflammation originating in the adipose tissue during obesity; however, the specific immunological mechanisms regulating the progression of NAFLD-associated fibrosis in the liver are unclear. To investigate the immunopathogenesis of NAFLD more completely, we investigated adipose dysfunctions, nonalcoholic steatohepatitis (NASH), and fibrosis in mice that develop polarized type 1 or type 2 immune responses. Unexpectedly, obese interleukin-10 (IL-10)/IL-4-deficient mice (type 1–polarized) were highly resistant to NASH. This protection was associated with an increased hepatic interferon-γ (IFN-γ) signature. Conversely, IFN-γ-deficient mice progressed rapidly to NASH with evidence of fibrosis dependent on transforming growth factor-β (TGF-β) and IL-13 signaling. Unlike increasing type 1 inflammation and the marked loss of eosinophils seen in expanding adipose tissue, progression of NASH was associated with increasing eosinophilic type 2 liver inflammation in mice and human patient biopsies. Finally, simultaneous inhibition of TGF-β and IL-13 signaling attenuated the fibrotic machinery more completely than TGF-β alone in NAFLD-associated fibrosis. Thus, although type 2 immunity maintains healthy metabolic signaling in adipose tissues, it exacerbates the progression of NAFLD collaboratively with TGF-β in the liver.

INTRODUCTION

Treating the individual sequelae of the metabolic syndrome associated with obesity and type 2 diabetes represents one way to alleviate the overall burden of disease. For example, nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, is now the most common form of chronic liver disease, is growing in prevalence, and will soon be the most common cause of liver transplantation in the United States (1–3). This is highlighted by a recent estimate that NAFLD affects 64 million people in the United States, with annual direct medical costs projected at $103 billion (4). Although a great deal of research has focused on obesity, metabolic alterations in disease, adipose tissue dysregulation, and the pathophysiology of NAFLD, the molecular and immunological mechanisms that facilitate the progression of NAFLD to nonalcoholic steatohepatitis (NASH) and cirrhosis remain much less clear (5). Because severe cirrhosis is the ultimate reason patients require liver transplant and the presence of fibrosis has been identified as the best predictor of clinical outcome and mortality (6, 7), a better understanding of the mechanisms that drive fibrogenesis in NAFLD could reveal novel treatment strategies and have a major impact on morbidity and mortality. However, progress in this area has been stymied by the absence of preclinical models that reliably reproduce inflammatory and fibrotic components seen in human disease (8, 9).

It is clear that the initiation and progression of NAFLD along its spectrum of severity are intricately associated with the inflammatory processes that are driven by chronic obesity. The initiation of dyslipidemia and lipogenesis in the liver is tied to insulin resistance and underlying adipose tissue inflammation. How the dysregulated immune response in the adipose tissue affects whole organism metabolism during homeostasis and obesity has been the topic of intense study. The emerging model suggests that homeostatic type 2 immunity in adipose tissues driven by interleukin-5 (IL-5), IL-4, and IL-13 production from innate type 2 lymphocytes, resident eosinophils, regulatory T cells, and alternatively activated macrophages maintains healthy metabolic signaling (10–15). During obesity, this set point is skewed toward type 1 inflammation with increasing production of IL-1β, tumor necrosis factor-α (TNF-α), IL-6, IL-12, and interferon-γ (IFN-γ), resulting in loss of local and then systemic insulin sensitivity and establishment of the chronic low-level type 1 inflammation that is the hallmark of the metabolic syndrome. Thus, prevailing models hold that the inflammation contributing to NASH, cirrhosis, and eventual liver failure is an extension of dysregulated adipose tissue inflammation (16).

Here, we investigated whether type 1 inflammatory response in obese mice directly contributes to the development of NAFLD. Using a variety of transgenic mice that develop highly polarized type 1 and type 2 immune responses, we examined whether the progression of high-fat diet (HFD)–induced NASH and fibrosis is influenced by changes in type 2 immunity. Surprisingly, our studies reveal a disconnect in how obesity and the loss of type 2 effector function in adipose tissues affects the progression of inflammation, NASH, and fibrosis in the liver. In contrast to its protective role in metabolism, adipose tissue homeostasis, and obesity, we show that the type 2 cytokine IL-13 collaborates with transforming growth factor-β (TGF-β) to drive liver fibrogenesis in HFD-induced obesity.
RESULTS

NASH and fibrosis develop late in mice fed an HFD

NASH and the accompanying development of hepatic fibrosis are complications linked to chronic obesity. To establish a timeline for the development of NASH and to determine the time point at which obese mice develop NASH with fibrosis, we subjected animals to HFD for 15 and 40 weeks and then examined them for the hallmark features of steatohepatitis. Mice on HFD for 15 weeks gained appreciable weight and developed hepatomegaly (Fig. 1A). Despite being steatotic, there were no detectable inflammation and hepatocyte damage at this early time point, as assessed by liver leukocyte numbers (Fig. 1B) and serum aminotransferase level (Fig. 1C). Oil Red O staining was modest at this time point compared with 40 weeks (Fig. 1D). We also did not detect staining for ballooning hepatocytes (Fig. 1E) or fibrosis by picrosirius red staining of liver sections (Fig. 1F, top). Nevertheless, after 40 weeks, mice on HFD displayed increases in leukocyte infiltration of the liver (Fig. 1B), with histological evidence of marked lobular inflammation (Fig. 1E, inset). Additionally, increases in aminotransferases in serum (Fig. 1C) suggested that chronic HFD led to substantial hepatocellular damage. This was characterized by hepatocellular macrosteatosis (Fig. 1D) and ballooning degeneration, identified through immunohistochemical and immunofluorescence staining for ubiquitin and hepatic loss of keratin 8/18 staining (Fig. 1E and fig. S1A). There was also significant pericellular zone 3 accumulation of collagen in the livers of mice fed an HFD (Fig. 1F, bottom) and increased expression (fig. S1B) and immunofluorescence staining for

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Fig. 1. NASH and fibrosis develop late in mice fed an HFD. Mice were maintained on a normal diet (filled symbols; ND) or a 60% kcal from fat diet (open symbols; HFD) for 15 (circles) or 40 (squares) weeks. (A) Body weights and liver weight and liver index (% of body weight) were collected (n = 10 to 20). Characteristics of NASH were measured including isolated liver leukocyte counts (n = 9 to 12) (B), serum ALT and aspartate aminotransferase (AST) (n = 9 to 10) (C), and steatosis by Oil Red O staining (D). Scale bars, 100 μm. (E) Liver sections were stained with keratin 8/18 and ubiquitin (Ub.) to assess hepatocyte ballooning and hematoxylin and eosin (H&E) for hepatic inflammation (inset showing example of lobular inflammation). Scale bars, 40 μm. (F) Picrosirius red staining was performed to assess fibrotic collagen deposition (scale bars, 200 μm) and viewed under polarized light to enhance visualization (inset scale bars, 200 μm). Adipose inflammation was assessed from Giemsa-stained sections from 40-week-treated animals (scale bars, 150 μm) (G), crown-like structures marked with arrows, expression of tnf (H) (n = 4 to 5), and flow cytometry analysis of Siglec-F⁺ adipose eosinophils among CD45 leukocytes (I) (n = 5 to 12). All data points represent a single mouse, and representative or pooled data from two or more independent experiments are shown (two-tailed t tests, n = 2 to 10; *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001).
smooth muscle actin (fig. S1C), which are defining features of NASH. This timeline is in agreement with previously published work, which reports NASH phenotypes present in wild-type (WT) mice on HFD by 34 weeks (17). As predicted, the NASH phenotype was accompanied by increases in adipose inflammation measured by histological immune infiltration (Fig. 1G), presence of crown-like structures (Fig. 1G, arrowheads), and increased tnf expression (Fig. 1H). Furthermore, the increased inflammation in adipose tissues was accompanied by a loss of eosiophilus as has been previously reported (Fig. 1I) (15). The increased inflammation in adipose tissue resulted in increased TGF-β expression and induction of a number of collagens and metalloproteinases; however, we did not detect development of substantial fibrosis by picrosirius red staining of adipose tissue at either time point (fig. S2). These studies demonstrate that obese mice are susceptible to the sequelae of NAFLD but only when chronically exposed to HFD. These data also support the model that progression of NAFLD in obesity is associated with a loss of type 2 homeostatic set points and expansion of inflammation in adipose tissues.

**Type 1 immunity drives metabolic disease but regulates progression of NAFLD**

To determine whether the dysregulated proinflammatory immune response in adipose tissues of obese mice contributes to the development and progression of NAFLD, we investigated whether primary drivers of type 2 immunity protect against NAFLD. We used IL-4−/− and double IL-10−/−/IL-4−/− mice, which were previously shown to develop increasingly polarized type 1 inflammation after sterile or infectious challenge (18, 19). Our goal with the IL-10−/−/IL-4−/− mice was to assess extreme type 1 inflammatory skewing rather than the specific contribution of IL-10 to NAFLD, which remains unclear despite being previously studied (20–25). The single IL-10−/− mice, in contrast to the IL-10−/−/IL-4−/− mice, develop more of a mixed T helper cell 1 (TH1)/TH2 response when challenged with various inflammatory stimuli, which may, in part, explain the contradictory results regarding IL-10 in NAFLD (18, 19). We observed consistently low and unchanging levels of IL-10 with disease in mice and in human microarray data (fig. S3). Thus, we hypothesized that the IL-4−/− and IL-10−/−/IL-4−/− genetically deficient mice would display a progressive worsening of metabolic homeostasis, obesity, adipose tissue dysregulation, and increased progression toward NASH and fibrosis because of their highly polarized type 1 inflammatory response.

When challenged with HFD for 15 weeks, IL-4−/− mice developed similar obesity as WT mice measured by body composition (Fig. 2A) and serum leptin levels (Fig. 2B). However, the IL-4−/− mice displayed slightly worse hepatomegaly (Fig. 2C), despite having similar liver triglycerides (Fig. 2D). Unexpectedly, IL-10−/−/IL-4−/− mice were significantly protected from hepatomegaly, increased serum aminotransferases, and their liver triglyceride content, and steatosis was markedly reduced relative to WT and IL-4−/− mice (Fig. 2, C to E). This was surprising because the IL-10−/−/IL-4−/− mice were similarly susceptible to obesity compared to WT mice as measured by body composition and serum leptin levels (Fig. 2, A and B). This protection from the features of NAFLD despite normal susceptibility to obesity was sustained as long as 40 weeks on HFD in the IL-10−/−/IL-4−/− mice (fig. S4). Perigonal adipose tissues were also analyzed for evidence of dysregulated proinflammatory mediator expression. All three groups showed marked increases in tnf and ccl2, as well as readily observable increases in histological adipose immune infiltration and abundant presence of crown-like structures when fed an HFD (Fig. 2, F and G). The switch toward type 1 inflammation in adipose tissues was also accompanied by a substantial reduction in the number of adipose tissue eosiophilus (Fig. 2H). Compared to WT mice, both genetically deficient animals displayed similar or increased inflammation in the adipose tissue, but the IL-10−/−/IL-4−/− mice were resistant to steatosis and NASH when placed on HFD as measured by hepatic triglyceride levels, serum aminotransferase levels, and histological evaluations. Together, these findings suggest that there are disconnects between the mechanisms driving inflammation and metabolic dysregulation in adipose tissues and the mechanisms driving development of NAFLD.

**Obese IFN-γ−/− mice develop accelerated NAFLD with fibrosis**

To elucidate the mechanism for the reduced development of NAFLD in IL-10−/−/IL-4−/− mice, we performed RNA sequencing (RNA-seq) analysis on whole liver tissue isolated from WT, IL-4−/−, and IL-10−/−/IL-4−/− mice on a normal diet or HFD for 15 weeks. Ingenuity Pathway Analysis of significantly altered genes between the genotypes on a normal diet or HFD [analysis of variance (ANOVA)] identified interferon-γ (ifng) as the most activated pathway among predicted upstream regulatory pathways at baseline in the IL-10−/−/IL-4−/− livers (P = 2.65 × 10−31). This pathway was down-regulated slightly in WT animals on HFD (z score = −1.362). In IL-4−/− mice, the IFN-γ pathway was down-regulated whether the mice were on a normal diet (z score = −3.805) or HFD (z score = −2.64). In contrast, the IFN-γ pathway was the highest predicted up-regulated pathway in IL-10−/−/IL-4−/− mice on a normal diet (z score = 7.678) and was only slightly reduced when the mice were fed the HFD (z score = −1.069) (Fig. 3A). The IFN-γ signature in IL-10−/−/IL-4−/− mice was further validated when we searched for genes that, according to their annotation, are positively linked with IFN-γ activity (Fig. 3A, heat map). These data suggest that elevated baseline hepatic levels of IFN-γ that are highly activated in IL-10−/−/IL-4−/−, but not in IL-4−/− mice, may be protective against NAFLD.

Because the IFN-γ pathway was such a strong predictor of resistance or susceptibility to NAFLD in the IL-4 and IL-10/IL-4 genetically deficient mice, we investigated whether IFN-γ plays an important regulatory role in the progression of NAFLD. IFN-γ−/− mice on HFD for 15 weeks had similar increases in body weight and fat mass as WT mice (Fig. 3B). However, the IFN-γ−/− mice displayed markedly increased hepatomegaly and significantly increased steatosis compared to WT animals when assessed by liver triglyceride content and Oil Red O staining (Fig. 3, B and C). In additional studies, we found increased NAFLD in IL-12−/− and IL-10−/−/IL-12−/− mice compared to WT mice but no differences in NAFLD between IL-12−/− and IL-10−/−/IL-12−/− mice, suggesting that increased presence of type 1 regulatory mechanisms driven by IL-12 and IFN-γ is ultimately responsible for the differences we observed in the IL-10−/−/IL-4−/− mice rather than a specific effect of IL-10 (fig. S5). We also observed areas of NASH-like fibrotic deposition in the IFN-γ−/− mice by picrosirius red staining, which were undetectable in WT livers at the early 15-week time point (Fig. 3C, bottom). Additionally, the IFN-γ−/− mice had evidence of increased liver damage as measured by serum alanine aminotransferase (ALT) (Fig. 3D). The early detection of fibrosis in IFN-γ−/− mice was also accompanied by increased expression of collagen3a1 (col3a1), alpha smooth muscle actin, and the extracellular matrix–associated protein perisinost, which is produced by activated myofibroblasts in response to TGF-β1 and IL-13 stimulation (Fig. 3D).
Given that progression to fibrosis was accelerated in obese IFN-\(\gamma\)\(^{-/-}\) mice, we also examined whether TGF-\(\beta\)1 activity was altered. Expression of \(Tgfb1\) was significantly increased in the livers of IFN-\(\gamma\)\(^{-/-}\) mice, as were tissue inhibitor of matrix metalloproteinases–1 (TIMP-1) and matrix metalloproteinase–2 (MMP-2), which are both regulated by activated TGF-\(\beta\)1 (Fig. 3E) (28, 29). Furthermore, we observed localization of nuclear phosphorylated Smad3 in a greater frequency and variety of cell types present in the livers of HFD-challenged IFN-\(\gamma\)\(^{-/-}\) mice (Fig. 3F).

Next, we analyzed adipose tissues for markers of inflammation and detected increased \(tnf\) and \(ccl2\) expression in the obese IFN-\(\gamma\)\(^{-/-}\) mice and a substantial decrease in the number of tissue eosinophils (fig. S6A). Nevertheless, despite these changes, we failed to detect any significant increase in gross histological inflammation in the adipose tissues of IFN-\(\gamma\)\(^{-/-}\), which contrasts with the findings obtained in the adipose tissues of obese IL-4\(^{-/-}\) mice (fig. S6B). The worsening NASH-associated fibrosis is likely explained, in part, by the increased TGF-\(\beta\)1 signature in the livers of IFN-\(\gamma\)\(^{-/-}\) mice relative to WT or IL-4\(^{-/-}\).
NASH-driven fibrosis is partly TGF-β-dependent

Although TGF-β activity has been documented in the livers of NAFLD/NASH patients and in animal models of NAFLD/NASH and proposed as a therapeutic target, detailed experimental manipulation of the pathway to determine its importance in NASH-associated fibrosis and immune activation has not been performed (30–33). Thus, to determine the role for TGF-β signaling in chronic HFD-induced NASH and to validate its potential as a therapeutic target in NASH, we placed WT mice on HFD for 40 weeks, and TGF-β activity was inhibited therapeutically during the final 4 weeks of the study. Compared to animals receiving control antibody (Ab) injections, the animals treated with a pan TGF-β blocking Ab did not display significant differences in body composition, hepatomegaly, or steatosis as measured by liver triglycerides (Fig. 4, A and B). When we analyzed histological fibrosis using picrosirius red staining, there was a modest yet significant decrease in characteristic NASH fibrosis quantified as fibrotic fraction in the mice receiving anti-TGF-β therapy after just 4 weeks (Fig. 4B). This was also associated with a decrease in expression of pro-coll3a1 and pro-coll1a1. However, TGF-β Ab blockade did not affect periostin or pro-coll6a1 mRNA expression (Fig. 4C). Periostin has been proposed as a biomarker of type 2 inflammation in asthma (34), and proposed as a biomarker of type 2 inflammation in asthma (34), and proposed as a biomarker of type 2 inflammation in asthma (34),
Accelerated NASH-driven fibrogenesis in obese IFN-γ−/− mice is characterized by eosinophilic inflammation during TGF-β blockade

We repeated the TGF-β blockade studies in both WT and IFN-γ−/− mice on HFD for 20 weeks to test whether the highly susceptible IFN-γ−/− mice would serve as a more rapid model of NASH and to further investigate the potential involvement of type 2 immunity in the progression of NAFLD. Once again, IFN-γ−/− mice developed severe hepatomegaly by 20 weeks, which contrasted with WT mice that displayed little evidence of advanced NAFLD at this time point. Despite variability in histological fibrosis at this time point assessed by picrosirius red staining (Fig. 5A), we detected significant increases in periostin and pro-col6a1 expression in mice treated with anti-TGF-β mAbs, confirming that the machinery driving fibrosis was substantially increased (Fig. 5B). Because IFN-γ is known to cross-regulate type 2 inflammation, we hypothesized that the increases in periostin and pro-col6a1 expression could be due to further dysregulation of cytokine responses in the livers of these mice.
which was validated by increases in IL-13 mRNA expression observed in both WT and IFN-γ−/− mice after TGF-β blockade (Fig. 5C).

To determine the functional impact of the enhanced type 2 response on the liver microenvironment, we examined ccl11 expression and quantified tissue eosinophils, which have been shown to promote inflammation and fibrosis. Both were increased in the livers of IFN-γ−/− mice and further exacerbated by TGF-β blockade (Fig. 5, C and D), but we were surprised to find a similar but less marked increase in eosinophils in WT mice as well, despite the fact that ccl11 expression was not increased in this group. This increase in eosinophils with TGF-β blockade was recapitulated in 40-week–treated WT mice (fig. S7A). The presence of tissue eosinophils was confirmed through histological analysis of Giemsa-stained liver sections (Fig. 5E) and immunofluorescence staining of tissue sections for Siglec-F+ cells (scale bars, 125 μm) (Fig. 5F). WT mice were maintained for 40 weeks on normal diet (filled symbols) or HFD (open symbols) and received 4 weeks of biweekly control Ig or anti-TGF-β (250 μg each). Liver weight and liver index were determined (n = 9 to 14), and fibrotic fraction was calculated from picrosirius red–stained liver sections (n = 3 to 12). (B) Expression of interstitial collagen genes. Periostin, IL-13, and CCL11 expression were quantified from whole liver tissue (n = 6 to 14). (C) Hepatic eosinophils were measured by flow cytometry analysis of Siglec-F+ cells among CD45 leukocytes (n = 7 to 9) (D). Eosinophils were observed in hepatic tissue by Giemsa-stained liver sections (scale bars, 50 μm) (E) and by immunofluorescence staining of tissue sections for Siglec-F+ cells (scale bars, 125 μm) (F). WT mice were maintained for 40 weeks on normal diet (filled symbols) or HFD (open symbols) and received 4 weeks of biweekly control Ig (circles), anti–TGF-β (squares), or combined anti–TGF-β and IL-13 mAbs (triangles) (250 μg each). (G) Expression of col3a1 and col6a1 from whole liver tissue were assessed by qPCR (n = 3 to 6). All data points represent a single mouse, and representative or pooled data from two or more independent experiments are shown (two-tailed t tests, n = 2 to 10; *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001). Fig. 5. Accelerated NASH-driven fibrogenesis in obese IFN-γ−/− mice is characterized by severe eosinophilic inflammation during TGF-β blockade. (A) WT (circles) and IFN-γ−/− (squares) mice were maintained for 20 weeks on normal diet (filled symbols) or HFD (open symbols) and received 4 weeks of biweekly control Ig or anti–TGF-β (250 μg each). Liver weight and liver index were determined (n = 9 to 14), and fibrotic fraction was calculated from picrosirius red–stained liver sections (n = 3 to 12). (B) Expression of interstitial collagen genes. Periostin, IL-13, and CCL11 expression were quantified from whole liver tissue (n = 6 to 14). (C) Hepatic eosinophils were measured by flow cytometry analysis of Siglec-F+ cells among CD45 leukocytes (n = 7 to 9) (D). Eosinophils were observed in hepatic tissue by Giemsa-stained liver sections (scale bars, 50 μm) (E) and by immunofluorescence staining of tissue sections for Siglec-F+ cells (scale bars, 125 μm) (F). WT mice were maintained for 40 weeks on normal diet (filled symbols) or HFD (open symbols) and received 4 weeks of biweekly control Ig (circles), anti–TGF-β (squares), or combined anti–TGF-β and IL-13 mAbs (triangles) (250 μg each). (G) Expression of col3a1 and col6a1 from whole liver tissue were assessed by qPCR (n = 3 to 6). All data points represent a single mouse, and representative or pooled data from two or more independent experiments are shown (two-tailed t tests, n = 2 to 10; *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001).
and macrosteatosis where fibrosis is observed. To further validate these observations and to confirm whether increases in the presence of type 2 inflammation are associated with the worsening NASH phenotype, we also assessed livers from WT animals on a chronic 40-week HFD for ccl11 expression and presence of eosinophils (fig. S7B) and detected significant increases in both measures.

Because IFN-γ negatively regulates both TGF-β and type 2 immunity, we wanted to verify that the marked protection observed in the livers of IL-10−/−/IL-4−/− mice was not accompanied by any induction of a profibrotic type 2 response. Confirming the evidence of hepatic protection in these mice, there was almost no induction of tgfbi or evidence of increased TGF-β or IL-13 activity measured by timp1 and postn expression or accumulation of eosinophils at 40 weeks on HFD. In contrast, all of these measures were increased in WT mice (fig. S8). This demonstrates that the increased IFN-γ signaling in the livers of the IL-10−/−/IL-4−/− mice is associated with protection and that activation of TGF-β or type 2 inflammation requires an additional stimulus such as steatosis or hepatic damage. We also examined the livers for the presence of tissue neutrophils and expression of neutrophil elastase, which we would have predicted to increase, based on models of type 1 inflammation serving as the major driver of NASH pathogenesis. Although we observed modest increases in expression of neutrophil elastase, particularly during TGF-β blockade, we found no significant changes in the frequencies of tissue neutrophils associated with HFD alone, TGF-β blockade, the absence of IFN-γ, or chronic HFD (fig. S7, C and D). As such, these observations are in stark contrast to the shifts in immune balance and loss of resident eosinophils seen in obese adipose tissue, indicating a significant divergence in the pathogenic mechanisms driving disease in each organ.

Finally, because of the increases in IL-13 production, up-regulation of IL-13-responsive genes, and increased presence of eosinophils, which both respond and contribute to IL-13 signaling, we investigated the combined roles of IL-13 and TGF-β in the progression of NASH-associated fibrosis. Returning to the 40-week model of NASH, we treated weight-matched animals therapeutically during the final 4 weeks with control, anti-TGF-β, or combined anti-TGF-β and anti–IL-13. Although the treatment regimen was too short to detect meaningful changes in disease, it was clear that the Abs were blocking fibrogenesis because we observed significant decreases in expression of both col3a1 and col6a1 with the dual blockade (Fig. 5G). This confirmed our hypothesis that col6a1 expression was being driven by IL-13 signaling because it was unaffected by the single TGF-β blockade. These observations demonstrate that TGF-β and IL-13 cooperatively drive fibrogenic pathways in the murine NASH liver.

AMLN diet–induced NASH recapitulates IFN-γ regulation and type 2 inflammation even with modest adipose inflammation

To determine the broad applicability of our findings in NASH, we repeated our analyses with WT and IFN-γ−/− knockout animals after 15 weeks on the amylin liver NASH model (AMLN) diet (40% fat, 20% fructose, and 2% cholesterol) (32, 36). This more severe model of NASH recapitulated our main findings, with IFN-γ−/− mice displaying similar increases in body weight (Fig. 6A) but having significantly increased hepatomegaly (Fig. 6A), expression of fibrotic markers (Fig. 6B), evidence of fibrosis (Fig. 6C), and hepatic damage (Fig. 6D). Furthermore, we found marked increases in induction of type 2 inflammation in both WT and IFN-γ−/− knockout animals measured by frequencies of IL-13 producing CD4 T cells and tissue expression of periostin (Fig. 6E), with significant increases in both measures in IFN-γ−/− mice. This was associated with increases in eosinophils and neutrophil accumulation in the livers of IFN-γ−/− mice (Fig. 6F). As suggested by the reduced weights of mice on the AMLN diet, reduction in eosinophils and increases in expression of TNF-α in the adipose tissue were less marked than what was observed with mice on HFD (Fig. 6G), suggesting that the induction of type 2 inflammation and fibrosis can occur somewhat independently from immune alterations in the adipose tissue.

**Human NASH is characterized by type 2 eosinophilic inflammation**

To confirm whether our NASH models were accurately recapitulating human disease and to determine whether our findings would be relevant to patients and potential therapeutics, we performed an analysis of NASH biopsies and publicly available microarrays from NASH patients. First, to determine whether there is a component of type 2 inflammation present in NASH patients, we assessed cytokine production from isolated and expanded intrahepatic lymphocytes (IHLs) after anti-CD3/CD28 restimulation. Compared to lymphocytes isolated from healthy controls, NASH-associated lymphocytes produced increased amounts of IL-4, IL-5, and IL-13 (Fig. 7A). Furthermore, when stratified by pathologist-assigned META VIR fibrosis scores, we observed increased type 2 cytokine production with worsening fibrosis, with significant increases observed in IL-5 and IL-4 between lower (F0-2) and higher (F3-4) fibrosis scores (Fig. 7B). Second, we determined that eosinophils were present in NASH patient livers by histological analysis and observed eosinophils present in similar patterns as seen in the murine livers in every NASH biopsy analyzed (n = 26) (Fig. 7C and fig. S10).

To further support eosinophils as an indicator of type 2 inflammation and potential contributor in progressive NASH, we performed an analysis of publicly available microarray data from healthy, steatotic, and NASH livers for genes with significant differential expression (37). Of the transcripts with significantly altered expression by steatosis or NASH, we identified 37 genes as either annotated or implicated in eosinophil function (38). Hierarchical clustering by samples and principal components analysis using this gene set separates the NASH from the healthy control and steatotic biopsies (Fig. 7D). Finally, we assessed potential mechanisms of eosinophil accumulation in the human microarray data. Although we did not detect significant changes in expression of the eotaxin genes in NASH microarrays, the eosinophil-associated chemoattractant genes ccl5, ccl23, and ptgds were all significantly up-regulated in NASH patients (fig. S9) (36, 38–43). These data indicate a previously unappreciated role for aspects of the type 2 response as a component of the inflammatory milieu contributing to NASH in patient livers and suggest that chronic HFD, AMLN diet, and the inflammatory pathway modifications we explore here reliably replicate this aspect of progression of NAFLD.

**DISCUSSION**

Research on the inflammation associated with NAFLD progression to NASH and cirrhosis has almost universally focused on components of the proinflammatory type 1 response including IL-1β, TNF-α, IL-6, IL-12, and Toll-like receptor (TLR) activation (16, 44). Here, we observed type 2 inflammatory components in both mouse and human NASH, suggesting a previously unappreciated role for type 2 inflammation in the progression of NASH to fibrosis, as IL-13 is a well-known contributor to...
progressive end-stage fibrosis in diseases of diverse etiology. The data presented here illustrate a much more mixed inflammatory milieu in the fatty liver than current models suggest, which could have significant implications for therapeutics that are based on altering the character of the inflammatory response in patients with NASH. Although eosinophils and other type 2–associated inflammatory cells play a protective role in adipose tissues, the findings here demonstrate that dysregulated type 2 inflammation contributes to the progression of steatosis and fibrosis in the liver (fig. S11).

We initiated these studies to better understand the protective and pathogenic roles of type 1 and type 2 inflammation in the pathogenesis of NAFLD, as recent studies have suggested that type 2 immunity plays a protective role in metabolic syndrome. Although we confirmed an important counterregulatory role for type 2 cytokines in adipose tissue inflammation (11, 12, 14, 15), our studies in IL-10−/−/IL-4−/− and IFN-γ−/− deficient animals revealed an unexpected protective role for some components of type 1 inflammation in NAFLD progression. Mechanistically, we show that targets of this IFN-γ regulation include the cytokines TGF-β and type 2–associated IL-13, which were dysregulated in the absence of IFN-γ. TGF-β has also previously been observed in NAFLD, and this pathway has been proposed as a possible therapeutic target for NASH-associated fibrosis (33, 45).

Fig. 6. AMLN diet–induced NASH recapitulates IFN-γ regulation and type 2 inflammation despite modest adipose inflammation. (A) WT (circles) and IFN-γ−/− (squares) mice were maintained for 15 weeks on normal diet (filled symbols) or AMLN diet (open symbols) and assessed for obesity by body weight (n = 5 to 8). NAFLD progression was assessed by liver weight and liver index, (A), expression of fibrotic markers col1a1, col3a1, and acta2 as assessed by qPCR (n = 5 to 7) (B), picrosirius red– and FAST green-stained liver sections from WT (top) and IFN-γ−/− (bottom) mice on a normal diet (left) and HFD (right) (C), and serum alkaline phosphatase and ALT levels (n = 4 to 8) (D). Type 2 response was measured by intracellular staining of hepatic CD4 T cells for IL-13 after PMA/ionomycin stimulation (n = 4 to 8), postn expression (n = 5 to 8) (E), and flow cytometry analysis of hepatic eosinophils and neutrophils (n = 5 to 8) (F). (G) Adipose inflammation was measured by flow cytometry analysis of eosinophils and tnf expression (n = 5 to 8). All data points represent a single mouse, and representative or pooled data from two or more independent experiments are shown (two-tailed t tests, n = 2 to 10; *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001).
To directly address the contribution of TGF-β to NAFLD-associated fibrosis, we performed a series of studies in which TGF-β activity was blocked therapeutically. Although a previous study characterized hepatocyte-specific deletion of Tgfbr2 in choline-deficient diet-induced NASH (45), our studies represent experimental intervention of TGF-β focusing on two critical targets of TGF-β signaling, fibroblasts and immune cells, without the caveat of eliminating potentially important TGF-β signaling during liver development. We found that TGF-β blockade alleviated profibrotic signaling pathways without having any detectable impact on average body weight, liver weight, or steatosis. However, in WT mice, we detected no changes in col6a1 and the matrix protein periostin, which have both been linked with IL-13–dependent inflammation and fibrosis (34, 35, 46). We also observed increases in il4 and chi3l3 expression after TGF-β blockade but little to no change in markers of type 1 inflammation, suggesting that TGF-β signaling regulates the emergence of type 2 inflammation in the fatty liver. These observations were even more striking in the highly susceptible IFN-γ−/− mice, in which TGF-β blockade led to marked increase in col6a1, col3a1, postn, il13, and ccl11 expression. We also observed increases in the number of eosinophils in the livers of the anti–TGF-β–treated IFN-γ−/− mice. Thus, although we showed that TGF-β serves as a mediator of fibrosis in NASH, these studies also uncovered an important collaborative role for type 2 immunity and IL-13 specifically in the progression of NASH and fibrosis.

The explanation for these findings remains unclear, although we hypothesize that activation of TGF-β and IL-13 signaling in the liver likely represents a feedback loop to repair hepatic damage induced by proinflammatory type 1 cytokines, lipid TLR ligands, dyslipidemia, and bacterial byproduct translocation during obesity-associated dysbiosis. Persistent activation or dysregulation of these repair pathways

![Fig. 7. Human NASH is characterized by type 2 eosinophilic inflammation.](http://stm.sciencemag.org/)

(A) Cytokine production from healthy (circles) (n = 2) and NASH (squares; red squares, HCV+ NASH patients) (n = 14) patient liver biopsies was measured from expanded intrahepatic lymphocytes after a CD3/CD28 restimulation for IL-4, IL-5, and IL-13. (B) Combined cytokine production stratified by METAVIR fibrosis score and representative anti–smooth muscle actin histology from F0-F2 (left) and F3-F4 (right) biopsies. (C) Eosinophils were observed in H&E-stained liver biopsies from patients (arrows). Scale bar, 25 μm. (D) Publicly available microarray data from healthy, steatotic, and NASH liver biopsies were analyzed for eosinophil-associated genes that demonstrated significantly altered expression, and hierarchical clustering by samples (left, heat map) and principal components analysis (right) was performed on the basis of this gene set. All data points represent a single patient.
can lead to the development of pathological fibrosis. This is supported by our detection of increased IHL production of IL-4, IL-13, and IL-5 and eosinophil accumulation in NASH patients. Eosinophils and type 2 cytokines have been implicated in both tissue repair and regeneration in models of acute liver injury (47, 48) and also in the development of fibrosis (35, 49, 50). Thus, given the hepatic injury and hepatomegaly associated with NAFLD and our observation of eosinophils in areas of macrosteatosis and fibrosis, we hypothesize that eosinophils, which are major producers of type 2 cytokines, including IL-13 (51, 52), may initially support liver repair and regeneration to maintain liver function and, at more chronic stages, may contribute to the development of fibrosis. The increasing pressure to repair the liver as obesity and metabolic syndrome become chronic may also explain the rapid loss of eosinophils from adipose tissues. Thus, worsening NAFLD could provide a feed-forward loop to further exacerbate adipose tissue dysregulation. Finally, our ability to stratify human NASH patients from normal and steatotic patients based on eosinophil-related gene expression and identification of eosinophils in 100% of NASH patient biopsy histologic sections analyzed suggests that the presence of eosinophils or eosinophil-derived mediators in liver biopsies may serve as a useful indicator of advancing NASH and the presence of type 2 inflammation more generally.

To directly test the contribution of type 2–associated inflammation to the activation of fibrogenic pathways in NASH, we blocked the key profibrotic cytokine IL-13 with a neutralizing mAb in combination with anti–TGF-β and found that IL-13, but not TGF-β, is critically required for the activation of col6a1 expression in the liver. Thus, IL-13 and TGF-β appear to activate distinct profibrotic mechanisms during the progression of NAFLD, suggesting that dual targeting of both core pathways may be needed to ameliorate the fibrotic progression of established fatty liver disease. Dual targeting may be particularly important because we observed the most marked increase in type 2 immunity when TGF-β was inhibited.

There are potential limitations to this study that may serve as future avenues of research or offer alternative interpretations of the data. With our use of global cytokine knockout animals, we focused on the liver and adipose tissue because these mice displayed similar susceptibility to obesity. However, we cannot exclude the possibility that the immune alterations were not having additional systemic effects, such as altering nutrient absorption in the intestine, modifying the microbiome, or modulating hormonal and nervous system signaling. Additionally, the number of NASH patient biopsies we assessed for IHL cytokine production (n = 14), the presence of eosinophils (n = 26), and microarray analysis (n = 16) was relatively small, making it uncertain whether type 2 inflammation serves as a universal driver of NASH or whether it only characterizes a particular subset of patients. However, given the similarities between the mouse and human data and the fact that the biopsies, histological analyses, and microarray data were gathered from independent research groups, there is substantial evidence supporting a critical role for type 2 immunity in the progression of NAFLD. Finally, although we were able to detect eosinophils in all NASH biopsies and identified transcriptional changes that were consistent with eosinophilic inflammation, we have not yet directly quantified eosinophils in human NASH or correlated their numbers with disease severity, which will be an important goal of future research.

In summary, we have identified a mixed inflammatory setting specific to the fatty liver containing a previously unappreciated component of type 2 immune activation marked by IL-13 and eosinophils, which collaborates with TGF-β through overlapping and independent mechanisms to drive fibrosis. We found evidence of this type 2 immune activation and eosinophil accumulation in both mouse and human NASH. These studies represent a paradigm shift in inflammatory characterization of steatohepatitis and have important implications for potential therapeutic interventions aimed at NASH and the metabolic syndrome.

**MATERIALS AND METHODS**

**Study design**

Our primary objective was to investigate the immunological contribution to NASH progression and fibrosis. To do this, we disrupted major regulatory cytokines or experimentally blocked relevant signaling molecules in diet-induced mouse models of progressive NAFLD/NASH. No statistical methods were used to predetermine sample size. Group sample size was chosen using records of variance in past experiments, and variance is similar between groups being statistically compared. All experiments were independently replicated two or more times. Samples or data points were excluded only in the case of a technical equipment or human error that caused a sample to be poorly controlled. Mice or samples were randomly assigned to experimental groups or processing orders. Group allocation was blinded for all mouse work when possible (such as administration of Abs, sample quantification and analysis, and pathology scoring). The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines in the EQUATOR (Enhancing the Quality and Transparency of Health Research) Network library were followed for this report. Primary data are located in table S1.

**Mice**

The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health Intramural Research Program, approved all of the experimental procedures (protocol “LPD 16E”). The program complies with all applicable provisions of the Animal Welfare Act (www.aphis.usda.gov/animal_welfare/downloads/awa/awa.pdf) and other federal statutes and regulations relating to animals. Six- to 8-week-old C57BL/6 male mice or knockout strains on C57BL/6 background were obtained from within the same barrier facility at Taconic Farms Inc. and thus were exposed to equivalent bacteria for microflora colonization. Mice were put on either a diet of 60% calories from lard fat diet from Harlan (TD.06414) or the AMLN diet with 40% fat, 20% fructose, and 2% cholesterol from Research Diets (D09100301). For therapies, 250 µg each of anti–TGF-β (BioXCell clone 1D11.16.8, catalog #BE0057), IL-13 (Genentech clone 262A-5-1), or control Ab (BioXCell clone MOPC-21, catalog #BE0083) was injected intraperitoneally twice weekly. Mouse body composition was assessed using 1H nuclear magnetic resonance spectroscopy (3-in-1 EchoMRI, Echo Medical Systems LTD) with assistance of O. Gavrilova and the Mouse Metabolism Core at National Institute of Diabetes and Digestive and Kidney Diseases. Mice were terminally anesthetized with sodium pentobarbital. All animals were housed under specific pathogen–free conditions at the National Institutes of Health in an American Association for the Accreditation of Laboratory Animal Care–approved facility.

**Immunofluorescence and immunohistological staining**

Liver tissue was snap-frozen immediately after perfusion using a CoolRack M96-ID freezing block on dry ice. Tissue was sectioned at 8 µm using a cryostat and maintained at −80°C until needed. Slides were removed from −80°C and immediately fixed for 15 min using
10% neutral buffered formalin. Sections were permeabilized for 20 min using 0.2% Triton X-100 phosphate-buffered saline (PBST). Sections were then blocked with 2% bovine serum albumin (BSA) PBST for 30 min. Sections were washed three times with PBST for 5 min each. Primary Abs were diluted in PBST 2% BSA and incubated with sections for 2 hours at room temperature. Sections were rinsed three times with PBST for 5 min each. Secondary antibodies were diluted in PBST 2% BSA and incubated with sections for 1 hour at room temperature. Sections were rinsed once with PBST for 5 min. Sections were then stained with 300 nM DAPI in PBST for 3 min. Sections were then blocked with 2% bovine serum albumin (BSA) PBST 2% BSA and incubated with sections for 1 hour at room temperature. Sections were rinsed three times with PBST for 5 min each. Additional blocking of endogenous enzymes was performed (Dako enzyme block). Keratin 8/18 was visualized by secondary staining with goat anti-rabbit horseradish peroxidase (HRP) (ab97057) developed with DAB substrate kit (ab64238). This was followed by visualization of secondary staining with Vina Green Chromogen (BRR807A).

Fibrosis quantification
Picrosirius red–stained cross sections of three liver lobes from each mouse were imaged under polarized light. Fibrotic area was calculated by measuring total area of liver with the presence of NAS–specific collagen deposition quantified using a Fiji image analysis software (ImageJ) and reported as fibrotic fraction.

Hepatic triglyceride measurement
Relative liver lipid was assessed by measuring glycerol content of hydrolyzed liver tissue as a surrogate of triglyceride accumulation (53–55). Briefly, glycerol was isolated from 50 to 100 mg of liver saponified by addition of ethanol KOH (two parts EtOH and one part 30% KOH) overnight at 55°C followed by addition of 1:1 water and EtOH. One molar MgCl₂ was added to precipitate fatty acids, and samples were spun down. Cleared supernatants were assayed for glycerol content with colorimetric Free Glycerol Reagent (Sigma-Aldrich, F6428) relative to a glycerol standard curve (Sigma-Aldrich, G7793), and liver triglyceride content was calculated.

Human NASH cytokine quantification
After informed consent, fresh human liver biopsy specimens were obtained from patients undergoing diagnostic liver biopsies at the Hepatology Clinic of the Centre Hospitalier de l’Université de Montréal. This study was approved by the institutional ethics committee (protocol SL09.228), and all experiments were performed in accordance with the Declaration of Helsinki. IHLs were isolated by enzymatic digestion with collagenase type 2 (100 IU/ml) ( Worthington) of the liver biopsy followed by mechanical dissociation through a 70-μm filter. IHLs were expanded for 15 days in 24-well plates with 2 × 10⁶ autologous irradiated (3000 gray) peripheral blood mononuclear cells as feeder cells in the presence of IL-2 (50 IU/ml) (R&D Systems) and anti-CD3 (1 μg/ml) (BD Biosciences clone UCHT1). Expanded IHLs (2 × 10⁶/ml) were restimulated for 3 days with anti-CD3/CD28 (1 μg/ml). Cytokine production was quantified from supernatants collected at day 3 diluted 1:2 by LEGENDplex analysis for Human Th cytokines (BioLegend) following the manufacturer’s instructions. LEGENDplex data were acquired on a standard LSR II instrument (BD Biosciences) using the FACSDIVA software version 8 and analyzed using LEGENDplex software version 7 (BioLegend).

Murine gene expression analyses
Lever and perigonadal adipose tissues were homogenized in TRIzol Reagent (Life Technologies) with Precellys 24 (Bertin Technologies). Total RNA was extracted with chloroform using a MagMAX-96 Total RNA Isolation Kit (Qiagen) and reverse-transcribed to complementary DNA using SuperScript II Reverse Transcriptase (Life Technologies). Gene expression was quantified using Power SYBR Green PCR Master Mix (Applied Biosystems) by reverse transcription polymerase chain reaction (PCR) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Gene expression is described relative to RPLP2 mRNA levels in naïve liver and perigonadal adipose tissues. Genes with National Center for Biotechnology Information GenBank accession codes are as follows: Rplp2 in mice were analyzed using a mouse reference sequence transcriptome (2015-0804) with the mouse mm10 genome with TopHat2 and mapped to the mouse transcriptome using the mouse mm10 genome with TopHat2 and mapped to the mouse transcriptome.

Human NASH microarray analysis
RNA isolated as described above was submitted to the National Institute of Allergy and Infectious Diseases Research Technologies Branch, where RNA-seq was performed. Illumina NextSeq 500 reads were aligned to the mouse mm10 genome with TopHat2 and mapped to the mouse reference sequence transcriptome (2015-0804) with the expectation-maximization algorithm in Partek Flow (build 40.15.0702). The nCounter Analysis System (NanoString Technologies) was used for gene expression analysis from RNA isolated from adipose tissue. RNA-seq data have been uploaded to the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) with accession number GSE95428. Previously
published and publicly available human microarrays from clinically defined normal, steatotic, and NASH patient livers were obtained from the ArrayExpress database (accession E-MEXP-3291). Subsequent analyses were performed using TM4 MeV microarray software suite. Welch’s t tests were used to generate volcano plots ($P < 0.05$) from which list subsets were generated by using fold difference cutoffs. Additional ANOVA tests were carried out between groups. Fold change values were uploaded to the Ingenuity Pathway Analysis software (Qiagen) to determine potential upstream regulators.

**Data availability**

Accession codes for murine gene expression analysis are provided above with primers. Mouse RNA-seq and human NASH microarray accession numbers are provided above.

**Statistical analysis**

Prism (version 6 and 7; GraphPad) was used for statistical analysis and graphing. Mann-Whitney or t test was used to determine significant differences. Data sets were compared with a two-tailed t test, and differences were considered significant if $P$ values were $<0.05$. Error bars represent SD, where shown.

**SUPPLEMENTARY MATERIALS**

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Materials and Methods

Fig S1. Chronic HFD-induced NASH is associated with hepatic ballooning and activation of fibroblasts.

Fig S2. Analysis of adipose tissue fibrotic pathways and collagen deposition.

Fig S3. IL-10 in mouse and human NASH.

Fig S4. NAFLD/NASH in 40-week HFD-challenged WT, IL-4, and IL-10/IL-4 knockout mice.

Fig S5. Comparison of IL-10 versus IL-12 deletion in NAFLD progression.

Fig S6. Chronic HFD-induced NASH is associated with hepatic ballooning and activation of fibroblasts.

Fig S7. Presence of cellular mediators of inflammation in NAFLD/NASH.

Fig S8. Collagen- and eosinophil-associated chemoattractant expression alterations in patient biopsies.

Fig S9. Collagen- and eosinophil-associated chemoattractant expression alterations in patient biopsies.

Fig S10. Representative histological eosinophils in NASH patient biopsies.

Fig S11. Inflammatory pathways in NASH progression.

Table S1. Primary data.

**REFERENCES AND NOTES**


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Competing interests: J.R.A. is an employee of Genentech Inc. The other authors declare that they have no competing interests. Data and materials availability: RNA-seq data have been uploaded to the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) with accession number GSE95428. Previously published and publicly available human microarrays were obtained from the ArrayExpress database (accession E-MEXP-3291).

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Type 2 immunity is protective in metabolic disease but exacerbates NAFLD collaboratively with TGF-β


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Opposing cytokines in obesity and fibrosis

Polarized cytokine networks can drive immunopathogenesis forward. Obesity that leads to liver fibrosis involves type 1 cytokines, so Hart et al. expected obese mice prone to type 1 cytokine responses to experience more pronounced fibrosis. Instead, they saw that these mice were resistant to steatohepatitis. Fibrotic livers from mice and human biopsies showed type 2 inflammation and recruitment of eosinophils, unlike the inflammation observed in the adipose tissue during obesity. These findings reveal that cytokine activity that is beneficial for the homeostasis of one tissue can be detrimental to another.

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