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*J Immunol* 2008; 181:4656-4665; doi: 10.4049/jimmunol.181.7.4656

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Novel Role of IL-13 in Fibrosis Induced by Nonalcoholic Steatohepatitis and Its Amelioration by IL-13R-Directed Cytotoxin in a Rat Model

Takeshi Shimamura,* Toshio Fujisawa,* Syed R. Husain,* Mitomu Kioi,* Atsushi Nakajima, † and Raj K. Puri*1

Nonalcoholic steatohepatitis (NASH), the most common cause of chronic liver disease in North America (1–3). It is estimated that 3% of US population has nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis in up to 20% of patients (2, 4). Advanced age, obesity, insulin resistance, hypertension, and diabetes have been associated with a higher risk of developing cirrhosis in NASH cases (5, 6). Given the current epidemic of obesity, particularly in children, it has been reported that the national health care burden related to cirrhosis due to NASH will continue to increase (7, 8). Genetic factors including hereditary hemochromatosis, angiotensinogen, and TGF-β1 genes have been also associated with progression of fibrosis to NASH (9, 10).

The end results of many inflammatory and tissue repair responses are the development of fibrosis (11, 12). Fibrosis is of particular concern in numerous persistent inflammatory diseases driven by Th2 responses leading to active fibrogenesis, including those resulting from pathogens such as Leishmania donovani and Schistosoma mansoni parasites and hepatitis viruses (9, 12–14).

IL-13 is a Th2 cytokine that plays a central role in various inflammatory diseases (15). IL-13 seems to induce tissue fibrosis by stimulating and activating TGF-β1 (16). IL-13 binds to two known chains, IL-13Rα1 and IL-13Rα2. IL-13Rα1 chain is a low-affinity receptor that forms a heterodimer with IL-4Rα chain to form a high-affinity IL-13R and mediate signal transduction through the JAK-STAT-6 pathway (17, 18). IL-13Rα2, on the other hand, binds IL-13 with high affinity and was not found to mediate signaling even though it was internalized after binding to IL-13 (19). IL-13Rα2 was shown to act as a decoy receptor in murine system (9, 20). We recently reported that IL-13 can signal through IL-13Rα2, in a STAT-6-independent manner, in murine macrophage cell line and that IL-13 is involved in fibrosis through the TGF-β1 pathway (11). However, it is not known whether IL-13 and its receptor are involved in NASH.

Hepatic stellate cells (HSC) play a central role in the development and resolution of liver fibrosis (21–23). Several types of cytokines, e.g., IL-6, IFN-γ, TGF-β1, TNF-α, endothelin-1, and platelet-derived growth factor, which regulate the inflammatory response to injury, cause HSC transdifferentiation from the quiescent phenotype to the activated myofibroblast-like (α-smooth muscle actin (α-SMA)-expressing) phenotype (9). HSCs are responsible for the majority of extracellular protein deposition in liver fibrosis, and recovery from established fibrosis can occur through the apoptosis of HSC and subsequent reduction in liver collagen (24, 25). Targeting activated HSC, not quiescent HSC, could be an appropriate strategy to eliminate established liver fibrosis.

Because IL-13R are overexpressed on cancer cells, we have developed a recombinant fusion protein, IL-13 cytokinin (IL13-PE38), composed of human IL-13 and a mutated form of Pseudomonas exotoxin to target these receptors (26). After binding to IL-13R on the cell surface, IL-13-PE38 prevents the initiation of protein synthesis, leading to cell death through necrotic and apoptotic pathways (27). IL-13-PE38 mediates antitumor effects in IL-13Rα2-positive cancer cells in vitro and animal models of human cancer (28). After the successful completion of several phase 1 and 2 clinical trials with IL-13-PE38 in patients with recurrent glioblastoma, a multicenter phase 3 clinical trial (PRECISE study) is now completed (29).

To determine whether IL-13 plays a role, we examined the expression of IL-13Rα2 in liver biopsy samples from subjects with

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NASH and fatty liver disease. We also examined whether HSCs express IL13Rα2, respond to IL-13 and IL13-PE38 mediates IL-13R-specific cytotoxicity to HSC. We developed an animal model of NASH induced by choline-deficient t-amino acid-defined (CDAA) diet (30) and examined the role of IL-13R in vivo on liver fibrosis after treating rats with IL13-PE38. Finally, we studied the mechanism of IL-13 induced fibrosis through the TGF-β pathway in activated HSCs.

Materials and Methods

Cell culture, reagents, tissue specimens, and serum samples

Hepatocytes and HSCs (ScienCell) were cultured in hepatocyte or HSC medium containing growth factors. The L9-0 cell line exhibiting human HSC characteristics was kindly provided by Human Science Cell Bank (Saitama, Japan). Clinical liver biopsy samples and sera from NASH, fatty liver, and normal pathologically diagnosed subjects were obtained from Yokohama City University Hospital (Yokohama, Japan). rIL13-PE38 was generated as previously described (26).

RT-PCR and real-time TaqMan RT-PCR

Total RNA was extracted from clinical samples and cell lines with TRIZOL reagent (Invitrogen) followed by treatment with DNase I (Promega). The total RNA was reverse transcribed as described earlier (31, 32). For neutralization of TGF-β activity, HSCs were preincubated with anti-TGF-β Abs (clone ID11; R&D Systems) for 1 h before the addition of IL-13.

Quantitative PCR reactions were performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems). cDNA (50 ng) was added to a reaction volume (30 μl) containing 1x TaqMan PCR master mix, IL-13Rα2-specific probe/primer set, and GPDH or β-actin-specific probe (5'-VIC, 3'-MGB)primers mix (Applied Biosystems). Gene expression was normalized to GPDH to calculate the fold change in gene expression.

Confocal microscopy

Tissue sections were deparaffinized by xylene and washed with various concentrations of ethanol and PBS. Sections were incubated with anti-α-SMA mAb (Calbiochem) or isotype control (IgG) followed by FITC-labeled secondary Ab and incubated with Cy3-conjugated anti-IL-13Rα2 Ab (clone ID11; R&D Systems) or isotype control (IgG) followed by FITC-labeled secondary Ab and analyzed by FACScan (BD Biosciences). HSCs were stimulated in the presence or absence of human TNF-α, TGF-β1, IL-4, or IL-13 alone or in their combinations for 48 h.

ELISA assay

TNF-α, TGF-β1, and IL-13 levels were measured in triplicate using ELISAs (Quantikine ELISA; R&D Systems) according to the manufacturer’s instructions (33).

Protein synthesis inhibition assay

The in vitro cytotoxic activity of IL13-PE38 was measured by the inhibition of protein synthesis as described earlier (31). All assays were done in quadruplicate, and the concentrations of IL13-PE38 causing 50% inhibition of protein synthesis (IC50) were calculated. The inhibition of protein synthesis is directly proportional to cell death.

Cell proliferation inhibition studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

HSCs were seeded in 96-well plates with cytokines for 48 h, washed with PBS three times, and treated with various concentrations of IL13-PE38 for 72 h. After treatment, cells were incubated with MTT (0.5 mg/ml; Sigma-Aldrich) in medium at 37°C for 2 h and then with isopropanol at room temperature for 1 h. The optical density was determined at 595 nm using Spectra Max 5 (Molecular Devices).

Animals

Male Fischer 344 rats, 6 wk of age and weighing 140–150 g, were maintained in a barrier facility on high-efficiency particulate air-filtered racks. All animal studies were conducted under an approved protocol in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. CDAA diet was obtained in pellets (Dyets), and its composition is described in Ref. 34. Normal diet group animals were fed a control (choline-supplemented t-amino acid-defined; CSAA) diet.

Treatment protocol

Twenty rats fed with CDAA diet were divided into control and IL13-PE38 treatment groups. IL13-PE38 (50 μg/kg) was administered i.p. three times on alternate days, and organs were collected 7 days posttreatment. Liver samples were harvested on wks 8 and 12 after starting the diet.

Histology and immunohistochemical examination

The right lobes of all rat livers in 5-μm-thick sections were fixed in 10% formalin for 24 h, embedded in paraffin, and then processed for Masson trichrome and Sirius red staining. The activated stellate cells were immunohistochemically assessed using IL-13Rα2 and α-SMA mAbs. To avoid nonspecific reaction by endogenous biotin and peroxidase in hepatocytes, the sections were incubated with Dako Envision Plus system (Dako) with diaminobenzidine chromogen and counterstained with hematoxylin. α-SMA- and Sirius red-positive areas in the liver were quantified using a Provis microscope (Olympus) equipped with a charge-coupled device camera, and subjected to computer-assisted image analysis with image J (National Institutes of Health, Bethesda, MD) software. Ten randomly selected different areas per specimen were analyzed. The area of Sirius red- and αSMA-positive cells was calculated as the percentage of the total area of the specimen.

Hydroxyproline assay

Liver collagen concentration was determined by measuring hydroxyproline protein content in liver samples using a modified method reported previously (35). Briefly, liver samples were homogenized in PBS, pH 7.4, and then digested in 6 N HCl (final concentration, 6 N) for 18 h at 110°C. After filtration of the hydrolysate through a 0.45-μm pore size Millipore filter, samples were evaporated in a rotary evaporator. Five microliters of citrate-acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 μl of chloramine-T solution (282 mg of chloramine-T, 2 ml of n-propanol, 2 ml of H2O, and 16 ml of citrate-acetate buffer) were added to samples and left at room temperature for 20 min. Next, 100 μl of Ehrlich’s solution (Sigma-Aldrich) were added to each sample, and the samples were incubated for 15 min at 65°C. Samples were cooled for 10 min and read at 550 nm using SpectraMax M5 (Molecular Devices). Hydroxyproline (Sigma-Aldrich) concentrations from 0 to 200 μg/ml were used to generate a standard curve.

Luciferase assay

A plasmid containing the human TGFβ1 promoter linked to luciferase reporter gene was provided by J. Nam (National Cancer Institute, National Institutes of Health). This plasmid contains −436/+55 bp flanking the transcription start site of the human TGFβ1 promoter. HSC (0.5 × 106 cells/ml) in a 96-well plate were transiently transfected with the luciferase reporter plasmid pTGFβ-lucifase (250 ng) and the pSV-β-galactosidase vector (25 ng; Promega) by overnight incubation with HVJ-E loaded with plasmid vector. The transfected cells were placed in medium containing 10% TGF-β1-depleted human serum and stimulated for 24 h. Cell lysates were analyzed for luciferase activity (Promega) and β-galactosidase activity (Applied Biosystems). Data are means ± SD of triplicate determinations from three independent experiments. β-Galactosidase activity was used for normalization of TGF-β1 promoter activity in the luciferase assay.

Statistical analysis

Cytokine serum levels in patients with NASH, fatty liver, and healthy donors were analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA. The fibrotic areas in tissue sections from control and IL13-PE38-treated groups were also analyzed by ANOVA.
fatty liver and normal liver specimens did not (Fig. 1A). Confocal microscopy showed IL-13Rα2 and α-SMA expression in sinusoidal lesions in the NASH liver samples and when both images were merged, IL-13Rα2 and α-SMA coexpressed in HSC (Fig. 1B). All 14 tissue samples showed histological evidence of fibrosis. In sharp contrast to and consistent with RT-PCR results, IL-13Rα2 expression was not detected in sinusoidal lesions of normal and fatty liver specimens (Fig. 1B). Because HSCs show strong α-SMA expression when trans-differentiated from the quiescent phenotype to the activated myofibroblast-like phenotype, these results indicate that IL-13Rα2 expression was also detected on the activated HSC, but not in quiescent HSC or hepatocytes.

TNF-α, TGF-β1, and IL-13 cytokines are increased in sera of patients with NASH but not in healthy subjects

As TNF-α, TGF-β1, and IL-13 are implicated in inflammation and fibrosis, we analyzed sera from 17 patients with NASH, 11 with fatty liver, and 15 healthy donors. Serum TNF-α, TGF-β1, and IL-13 levels were assayed in triplicate by ELISA. Each circle indicates the average of three different determinations. p values for significant difference between groups were determined by ANOVA and Student’s t test.
and individuals with fatty liver disease (2.53 ± 0.98 pg/ml; p < 0.001; Fig. 1C). Serum TGF-β1 levels were also significantly increased in patients with NASH (62.75 ± 27.13 pg/ml) compared with healthy controls (2.50 ± 3.20 pg/ml, p < 0.001), but not with fatty liver disease (103.38 ± 45.74 pg/ml) (Fig. 1C). Serum IL-13 levels almost doubled in patients with NASH (20.62 ± 8.22 pg/ml), and their combination were stained with anti-IL-13Rα2 mAb and analyzed by flow cytometry. Representative histograms from three separate experiments are shown.

**FIGURE 2.** Regulation of IL-13Rα2 expression in HSCs. A, Total RNA extracted from HSCs and hepatocytes stimulated by TNF-α (50 ng/ml), TGF-β1 (10 ng/ml), or their combination were analyzed for IL-13Rα2 mRNA expression by RT-PCR. Renal cell carcinoma cell line (PMRCC), GAPDH, and IL-2 served as positive, internal, and negative controls, respectively. B, IL-13Rα2 mRNA expression was quantified by quantitative RT-PCR. Columns represent means ± SE of triplicate determinations. C, HSCs cultured with TNF-α (50 ng/ml), TGF-β1 (10 ng/ml), and their combination were stained with anti-IL-13Rα2 mAb and analyzed by flow cytometry. Representative histograms from three separate experiments are shown.

**TNF-α and TGF-β1 up-regulate IL-13Rα2 expression in HSC**

Because TNF-α and TGF-β1 levels were elevated in serum of patients with NASH, we examined whether these cytokines enhanced the expression of IL-13Rα2 in HSC and normal hepatocytes cultured with TNF-α and/or TGF-β1. IL-13Rα2 mRNA expression was strongly induced by each cytokine. The combination of both cytokines further increased the mRNA band intensity compared with either cytokine alone. In contrast to HSC, IL-13Rα2 mRNA expression was not induced by these cytokines in normal hepatocytes (Fig. 2A). Similarly, type I collagen mRNA was also up-regulated by TNF-α and TGF-β1, and their combination showed the highest up-regulation. As expected, a renal cell carcinoma cell line (PMRCC), a positive control, did not express type I collagen. As a control, IL-2 did not up-regulate either IL-13Rα2 or collagen type I mRNA expression. The induction of IL-13Rα2 mRNA expression by cytokines was further confirmed by real-time RT-PCR analysis (Fig. 2B). Again, the
combination of both TNF-α and TGF-β1 caused the highest expression of IL-13Ra2 mRNA, and there was no up-regulation by IL-2.

The up-regulation of IL-13Ra2 chain was confirmed by flow cytometric analysis (Fig. 2C). Similar to mRNA, protein expression was up-regulated by TNF-α or TGF-β1, and the highest up-regulation was observed with both cytokines. IL-2 did not enhance IL-13Ra2 protein expression (data not shown). Consistent with a previous report, both IL-4 and IL-13 increased IL-13Ra2 mRNA expression and protein levels, but increase of IL-13Ra2 protein on the cell membrane was not observed (36). The combination of IL-4 and TNF-α or IL-13 and TNF-α did not further increase IL-13Ra2 protein expression compared with TNF-α alone (data not shown). Comparable results were obtained in the experiments using the HSC LI-90 cell line (data not shown).

Role of IL-13Ra2 and other receptor chains in IL-13 signaling in HSC

As increased IL-13 level in the serum and IL-13Ra2 expression in the liver specimens are detected in NASH patients, we examined whether IL-13 signal is activated in HSC expressing IL-13Ra2. In addition, we examined the expression and roles of IL-13Ra1 and IL-4Ra chains in HSCs. HSCs transfected with IL-13Ra2 expressed IL-13Ra2 mRNA and when stimulated with IL-13,
TGFB1 promoter activity was significantly induced. In contrast, TGFB1 promoter activity was not induced by IL-13 in IL-13Rα2-negative normal or mock vector-transfected HSCs (Fig. 3B). In contrast, TGFB-β1 induced TGFB1 promoter activity in both mock- and IL-13Rα2-transfected HSC. The mRNA of IL-13Rα1 and IL-4Rα chains were also expressed in mock- and IL13Rα2-transfected HSC cells; however, no difference in expression was observed between both types of cells (Fig. 3A). Thus, the IL-13Rα2 chain is predominantly involved in IL-13-induced TGFB1 promoter activation. ELISA confirmed the increased level of TGF-β1 production by the stimulation of IL-13 in IL-13Rα2-positive HSCs, whereas it was not detected in normal and mock vector-transfected HSC (Fig. 3C). IL-4 stimulation did not increase TGF-β1 production in both types of HSC, indicating specificity for IL-13 and IL-13Rα2. For a positive control, HSC mock- and IL-13Rα2-overexpressing HSCs were stimulated with TGF-β1. The stimulation of these cells with TGF-β1 did not show a significant difference in TGF-β1 production by either HSC mock (2274 ± 296.9 pg/ml)- or IL-13Rα2-transfected HSCs (2309 ± 172.5 pg/ml). Taken together, these results suggest that IL-13 signals through IL-13Rα2 but not in IL-13Rα2-negative HSCs and that the type II IL-4R complex does not participate in TGF-β1 production.

To confirm that IL-13/IL-13Rα2 signaling induces fibrosis through TGF-β1 production, we measured collagen type I mRNA in IL-13- and

FIGURE 4. Cytotoxicity and antiproliferative effects of IL13-PE38 in IL-13Rα2-positive HSCs and normal hepatocytes. A, HSCs (1 × 10⁴) not stimulated or stimulated with TNF-α (50 ng/ml), TGF-β1 (10 ng/ml), IL-2 (50 ng/ml), or the combination of TNF-α and TGF-β1 were incubated with various concentrations of IL13-PE38 (0–1000 ng/ml), and inhibition of protein synthesis was measured. Bars represent means ± SD of triplicate determinations, and the assay was repeated twice. B, HSCs (1 × 10⁴) and IL13Rα2-overexpressing HSCs were incubated with various concentrations of IL13-PE38. C, HSCs (1 × 10⁴) were not incubated or incubated with the combination of TNF-α (50 ng/ml) and TGF-β1 (10 ng/ml). MTT assay was performed for determination of antiproliferative activity of IL13-PE38. Bars represent means ± SD of triplicates. D, Protein synthesis measurement of hepatocytes incubated with IL13-PE38 (0–1000 ng/ml).

FIGURE 5. Expression of IL-13Rα2 in fibrotic areas but not in normal hepatocytes. Liver samples collected from CDAA diet-fed rats for 8 wk were stained with IL13Rα2 (A) and mouse IgG1 (B) Ab. Each section was stained by Masson’s trichrome technique. ×40.
TGF-β1-stimulated mock HSCs and IL-13Rα2-transfected HSCs. IL-13 stimulation was blocked by preincubation with anti-TGF-β Ab (Fig. 3D). This result showed that IL-13 increased collagen type I mRNA in IL-13Rα2-transfected HSCs and that anti-TGF-β Ab prevented the collagen type I induced by IL-13. In contrast, IL-13 and anti-TGF-β Ab did not affect collagen type I induction in mock HSCs. Therefore, in IL-13Rα2-positive HSC, IL-13 can induce collagen type I through the TGF-β pathway. Our results further indicate that the TGF-β1 level induced by IL-13 treatment was enough to produce collagen type I, one of the critical components of liver fibrosis.

**IL13-PE38 is cytotoxic to HSCs expressing IL-13Rα2 but not to HSC or hepatocytes devoid of IL-13Rα2**

Because TNF-α and TGF-β1 induced IL-13Rα2 expression in HSCs at both the mRNA and protein levels, we examined whether IL-13Rα2 are functional and whether these cells become targets for IL13-PE38. HSCs were least sensitive to the cytotoxic effect of IL13-PE38. However, HSCs stimulated with TNF-α, TGF-β1, or both cytokines become susceptible to IL13-PE38 (Fig. 4A). The IC50 values in TNF-α- and TGF-β1-treated cells and those of and both cytokines were 87, 104, and 12 ng/ml, respectively compared with >1000 ng/ml in control HSCs. Consistent with the lack of effect of IL-2 on IL-13Rα2 expression, IL-2-treated cells did not show cytotoxicity to IL13-PE38. The IL13-PE38 activity was neutralized by an excess of IL-13, indicating a receptor-specific effect (not shown). HSCs transfected with IL-13Rα2 showed the highest sensitivity to IL13-PE38 (IC50 0.94 ng/ml) compared with mock HSC (Fig. 4B) or normal hepatocytes (IC50 1000 ng/ml) (Fig. 4D). Protein synthesis inhibition results were confirmed by MTT assay. IL13-PE38 inhibited cell proliferation in a dose-dependent manner of HSCs stimulated by TNF-α plus TGF-β1 but not of normal untreated HSC (Fig. 4C). These data indicate that specific cytotoxicity and anti-cell proliferation by IL13-PE38 are mainly regulated through binding of the IL-13 portion of IL13-PE38 to the IL-13Rα2 chain.
IL13-PE38 eliminates IL-13Ra2-positive fibrotic cells and hepatic fibrosis in rat model of NASH

During the pathogenesis of human NASH, hepatic steatosis is followed by inflammation, oxidative damage, and fibrosis in liver (37). To simulate these clinical features of NASH, we fed rats a CDAA diet to induce liver fibrosis as reported previously (30, 34). Rats were fed CDAA diet for 8 and 12 wk, and then liver samples were analyzed for IL-13Ra2 expression by immunohistochemistry. Fibrotic livers stained with Masson’s trichrome showed strong moderate staining for IL-13Rα. Fibrotic livers stained with Masson’s trichrome showed strong moderate staining for IL-13Rα.

Normal CDAA diet 12 wk
No treatment (n = 4) 7.84 ± 1.24 (100) 7.14 ± 0.70 (100) 
IL13-PE38 (n = 4) 2.35 ± 0.45 (23.9) 2.12 ± 0.41∗ (29.7) 

α-SMA-positive area (%)

Rats were fed the CDAA diet for 8 or 12 wk. After treatment, rats were sacrificed, and livers were stained with α-SMA and Sirius red stain. α-SMA-positive and Sirius red-positive areas were measured as described in Materials and Methods.

IL-13Ra2 expression by immunohistochemistry was seen in hepatocytes, portal vein, and bile duct (Fig. 5A). Staining of similar sections with isotype control Ab did not show any specific staining (Fig. 5B).

Masson’s trichrome staining showed reduction of fibrotic areas (Fig. 6, B and D) in CDAA-fed rats for 8 and 12 wk and when treated with IL13-PE38 (50 μg/kg on alternate days for three treatments on wks 9 and 13), whereas untreated control rat livers showed distinct fibrosis (Fig. 6, A and C). Livers from rats fed with CDAA diet showed extensive accumulation of fibrosis as confirmed by Sirius red staining (not shown). Quantitative analysis showed that the average Sirius red-positive area in liver sections of CDAA-fed rats for 8 and 12 wk was reduced by 73% (from 7.21% to 1.93%) and 76% (from 9.84% to 2.35%), respectively, by IL13-PE38 (Table I). The average of α-SMA-stained areas was also reduced by 79% and 70% in the 8- and 12-wk models, respectively (Table I). Thus, IL13-PE38 significantly (p < 0.01) reduced the fibrosis area in the livers of CDAA-fed rats. To further confirm these results, we performed quantitative hydroxyproline assays in rat livers fed with CDAA and control CSAA diets. As shown in Fig. 6E, the level of hydroxyproline in livers of CDAA diet fed rats was significantly increased at both 8 wk and 12 wk of feeding compared with control diet (CSAA)-fed rats (p < 0.001). The IL13-PE38 treatment of CDAA-fed rats significantly decreased the levels of hydroxyproline at both time points (p < 0.001). These results suggest that IL13-PE38 decreased the liver fibrosis and support our histological analysis of fibrosis in NASH liver (Fig. 6, A–D).

Serum chemical changes and organ histology in control and CDAA-fed rats and after IL13-PE38 treatment

There was no significant difference between the CDAA- and normal diet (CSAA)-fed rats in terms of the total amount of calories consumed. Body weights of rats in both groups were also similar (data not shown). Consistent with NASH-induced pathology, rats fed with the CDAA diet for 8 wk showed an increased serum alanine aminotransferase, aspartate aminotransferase, and alkaline phospholipid level of 627 ± 99 U/L, 500 ± 68 U/L, and 492 ± 39 U/L, compared with 64 ± 11 U/L, 98 ± 22 U/L, and 165 ± 32 U/L, in rats fed with CSAA diet, respectively. However, rats fed with the CDAA diet for 8 wk and then treated with IL13-PE38, showed reversal of enzyme elevation normalizing to baseline levels within 2 wk of treatment (data not shown).

Histological examination at necropsy did not reveal organ toxicity in heart, lung, kidney, and spleen of IL13-PE38-treated rats with NASH (data not shown).

Discussion

We demonstrate that IL-13Ra2 is expressed in sinusoidal lesions of liver from patients with NASH. These receptors coexpressed with α-SMA in HSC. In vitro data show that IL-13 is functional in HSCs and it signals through IL-13Ra2 leading to activation of TGFB1 promoter activity, TGF-β1 production and fibrosis. Interestingly, IL13-PE38 was highly cytotoxic to activated HSCs expressing IL-13Ra2 induced by TNF-α or TGF-β1, but not to quiescent IL-13Ra2-negative HSCs or hepatocytes. CDAA-fed rats developed NASH with prominent areas of fibrosis and fibrotic cells expressing IL-13Ra2. Treatment of these animals with IL13-PE38 significantly decreased fibrosis.

IL-13Ra2 expression has been reported on fibroblasts in human fibrotic diseases including idiopathic interstitial pneumonia and schistosomiasis (38, 39). We demonstrate for the first time that the IL-13Ra2 is expressed in liver specimens obtained from patients with NASH. Resting HSC fibroblasts did not express IL-13Ra2; however, TGF-β1 and TNF-α induced high levels of IL-13Ra2. This is consistent with a high level of expression of IL-13Ra2 in activated fibroblasts in livers with NASH. IL-13 mediates tissue fibrosis by regulating the production and activation of TGF-β1, a known mediator of fibrosis (16). Using an IL-13-transgenic mouse that overexpresses IL-13 in the lung, Elias and colleagues showed that IL-13 is a potent inducer of matrix metalloproteinase-9 and TGF-β1. They also showed that when TGF-β1 activity is neutralized, collagen deposition in IL-13-transgenic mice is substantially decreased indicating a direct functional link between IL-13 and TGF-β1. Fichtner-Feigl et al. (11) showed that IL-13 induces TGF-β1 promoter and secretion in hemopoietic cells through IL-13Ra2 and leading to fibrosis. However, in the context of schistosomiasis and helminth parasitic diseases, IL-13 can induce fibrosis in the absence of TGF-β1 and in this situation overexpressed IL-13Ra2 acts as a soluble decoy receptor that decreases fibrosis (40). In this case, alternatively activated macrophages (M2) induced by IL-13 in vivo may play a critical role in fibrogenesis; however, in fibrosis induced by silica, M2 cells are involved in the early inflammatory stage of silicosis and establishment of the fibrotic process is not associated with M2 polarization (41). In addition, as IL-13Ra2 is overexpressed in M2 macrophages, it is possible that Kupffer cells may also be involved in IL-13Ra2 expression in NASH liver.

Table I. Effect of IL13-PE38 on α-SMA-positive and Sirius red-positive area

<table>
<thead>
<tr>
<th>Treatment (n = no. of rats)</th>
<th>Sirius Red-positive Area (%)</th>
<th>α-SMA-positive Area (%)</th>
</tr>
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<tbody>
<tr>
<td>CDAA diet 8 wk</td>
<td></td>
<td></td>
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<tr>
<td>No treatment (n = 4)</td>
<td>7.21 ± 0.67 (100)</td>
<td>5.87 ± 0.55 (100)</td>
</tr>
<tr>
<td>IL13-PE38 (n = 4)</td>
<td>1.93 ± 0.45∗ (26.8)</td>
<td>1.23 ± 0.38∗ (21.0)</td>
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<tr>
<td>Normal CDAA diet 12 wk</td>
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<td></td>
</tr>
<tr>
<td>No treatment (n = 4)</td>
<td>9.84 ± 1.24 (100)</td>
<td>7.14 ± 0.70 (100)</td>
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<tr>
<td>IL13-PE38 (n = 4)</td>
<td>2.35 ± 0.45∗ (23.9)</td>
<td>2.12 ± 0.41∗ (29.7)</td>
</tr>
</tbody>
</table>

a Rats were fed the CDAA diet for 8 or 12 wk. After treatment, rats were sacrificed, and livers were stained with α-SMA and Sirius red stain. α-SMA-positive and Sirius red-positive areas were measured as described in Materials and Methods.

b Mean ± SD. Numbers in parentheses, percent.

c p < 0.01 vs no treatment group.
Although IL-13-PE38 mediated remarkable antifibrotic effects, no visible toxicity or features such as weight loss and inactivity or lethargy were observed in rats receiving treatment. Previous IL13-PE38 toxicity studies have reported that none of the mice and rats showed any change in hepatic transaminases, hematological toxicity, or vascular leak syndrome at maximum tolerated doses (28). Similar study in cynomolgus monkeys after i.v. injection of IL13-PE38 (50 μg/kg for 5 days) caused reversible elevation of hepatic transaminases and creatinine kinase with subsequent decline to normal levels. In the current study, IL13-PE38 normalized the elevated liver enzymes caused by fibrosis and caused no histological changes in vital organs.

In conclusion, HSCs in NASH liver specimens express high levels of functional IL-13Rα2, respond to IL-13, induce TGFβ1 promoter activity, and cause TGFβ1 production. Because IL-13Rα2-positive cells were diminished by IL13-PE38 treatment, it may be an important therapeutic target for the treatment of TGFβ1-mediated fibrosis such as NASH. Therefore, further studies should be performed to explore the potential of IL13-PE38 in elimination of fibrosis and treatment of patients with NASH.

Acknowledgments
We thank Drs. Ramjay Vatsan and Andrew Byrnes for critical reading of the manuscript. We are grateful to Pamela Dover for technical help, procuring reagents, and general support for these studies.

Disclosures
The authors have no financial conflict of interest.

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