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IL-13 Activates a Mechanism of Tissue Fibrosis That Is Completely TGF-β Independent

Mallika Kaviratne,* Matthias Hesse,[1]* Mary Leusink,* Allen W. Cheever,[†] Stephen J. Davies,[‡] James H. McKerrow,[‡] Lalage M. Wakefield,[§] John J. Letterio,[§] and Thomas A. Wynn[2]*

Fibrosis is a characteristic feature in the pathogenesis of a wide spectrum of diseases. Recently, it was suggested that IL-13-dependent fibrosis develops through a TGF-β1 and matrix metalloproteinase-9-dependent (MMP-9) mechanism. However, the significance of this pathway in a natural disorder of fibrosis was not investigated. In this study, we examined the role of TGF-β in IL-13-dependent liver fibrosis caused by *Schistosoma mansoni* infection. Infected IL-13$^{-/-}$ mice showed an almost complete abrogation of fibrosis despite continued and undiminished production of TGF-β1. Although MMP-9 activity was implicated in the IL-13 pathway, MMP-9$^{-/-}$ mice displayed no reduction in fibrosis, even when chronically infected. To directly test the requirement for TGF-β, studies were also performed with neutralizing anti-TGF-β Abs, soluble antagonists (soluble TGF-βR-Fc), and Tg mice (Smad3$^{-/-}$ and TGF-βRII-Fc Tg) that have disruptions in all or part of the TGF-β signaling cascade. In all cases, fibrosis developed normally and with kinetics similar to wild-type mice. Production of IL-13 was also unaffected. Finally, several genes, including interstitial collagens, several MMPs, and tissue inhibitors of metalloprotease-1 were up-regulated in TGF-β1$^{-/-}$ mice by IL-13, demonstrating that IL-13 activates the fibrogenic machinery directly. Together, these studies provide unequivocal evidence of a pathway of fibrogenesis that is IL-13 dependent but TGF-β1 independent, illustrating the importance of targeting IL-13 directly in the treatment of infection-induced fibrosis. *The Journal of Immunology,* **2004, 173: 4020–4029.**

Fibrosis is a characteristic feature in the pathogenesis of a wide spectrum of diseases, including pulmonary fibrotic disorders, progressive systemic sclerosis, renal disease, and liver cirrhosis. Fibrosis is a normal consequence of tissue injury and chronic inflammation, characterized by the accumulation and activation of excessive numbers of fibroblasts, deposition of extracellular matrix (ECM)[3] proteins such as collagen, and distortion of normal tissue architecture. Although fibrosis typically begins as a part of wound healing responses, excessive accumulation of collagen and other ECM components during chronic inflammation can lead to the destruction of normal tissue architecture and loss of function. Thus, it is a major cause of morbidity and mortality worldwide (1, 2).

The mechanisms of fibrosis have been studied extensively in the murine model of schistosomiasis by our lab and others. Female schistosomes living in mesenteric veins produce hundreds of eggs a day, a subset of which get trapped in host tissues resulting in circumoval granulomas and fibrosis, the major pathological manifestations of the disease. Fibrosis is less severe in the earliest phase of egg deposition when Th1-associated cytokines are present, while a few weeks later the Th2 cytokines IL-4, IL-5, and IL-13 dominate and liver fibrosis increases dramatically. Recent studies aimed at dissecting the functional contributions of the Th1/Th2 cytokines in schistosomiasis identified IL-13 as an indispensable mediator of fibrosis (3, 4). Although the average size of egg-induced granulomas was unaltered in IL-13-deficient mice, the marked fibrotic changes were almost completely ablated in the absence of IL-13 even after several months of infection (5).

Interestingly, it was recently suggested that IL-13 mediates its effects by regulating the production and activation of TGF-β, a known mediator of fibrosis (6). Using a CC10-IL-13 transgenic (Tg) mouse that over-expresses IL-13 in the lung, Elias and colleagues (6, 7) showed that IL-13 is a potent inducer of matrix metalloproteinase (MMP)-9 and TGF-β1 expression, and that the activation of TGF-β1 is mediated by a MMP-9-dependent mechanism. Moreover, they showed that when TGF-β activity is neutralized, collagen deposition in the lungs of the IL-13 Tg mice is substantially decreased. Thus, these studies indicate a direct functional link between IL-13 and TGF-β.

There are three isotypes of TGF-β in mammals, TGF-β1, -2, and -3, all exhibiting similar biological activity. However, TGF-β1 has been the most widely studied in the context of fibrogenesis. Several cell types including T cells, macrophages, fibroblasts, and epithelial cells produce TGF-β. It is stored inside the cell as a disulfide-bonded homodimer noncovalently bound to a latency-associated protein that keeps TGF-β inactive. Binding of the cytokine to its receptor requires dissociation of the latency-associated protein, a process catalyzed in vivo by a number of cleaving agents including plasmin, cathepsins, calpain, thrombospondin, and matrix metalloproteinases (8, 9). Once activated, the functions of TGF-β are controlled by a downstream signaling pathway that begins with the phosphorylation of the type II TGF-βR in response

*Immunopathogenesis Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; [†]Biomedical Research Institute, Rockville, MD 20852; [‡]Tropical Disease Research Unit, Department of Pathology, Veterans Affairs Medical Center, San Francisco, CA 94143; and [§]Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, Bethesda, MD 20892

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[1] Current address: Cornell University Veterinary College, Veterinary Medical Center /C5 147, Ithaca, NY 14850.

[2] Address correspondence to Dr. Thomas A. Wynn, Immunopathogenesis Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 50, Room 6154, MSC 8003, Bethesda, MD 20892. E-mail address: twynn@niaid.nih.gov

[3] Abbreviations used in this paper: ECM, extracellular matrix; Tg, transgenic; SEA, soluble egg antigen; p.i., postinfection; MLN, mesenteric lymph node; SWAP, soluble worm Ag preparation; WT, wild type; rMuIL-13, recombinant murine IL-13; KO, knockout; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloprotease.

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to TGF-β binding. Subsequently, the TGF-βR phosphorylates the type 1 receptor serine/threonine kinase. The activated type 1 receptor in turn phosphorylates the proteins, Smad2 and -3 (primary cytoplasmic mediators of TGF-β1 signaling), which form multimers with Smad4 that translocate into the nucleus. Once in the nucleus, the Smad complexes bind a well-defined Smad-response element (10), which triggers gene activation, including the production of ECM proteins such as collagen.

Thus, a step-wise linear pathway of fibrosis has been proposed wherein IL-13 induces and activates TGF-β1 through an MMP-9-dependent mechanism. Nevertheless, the relative importance of this pathway in a natural model of fibrosis has not been previously investigated. Moreover, given that many anti-fibrotic therapies are currently focused on inhibiting TGF-β (11–13), it is important to determine whether the collagen-inducing activity of IL-13 is mediated solely by the downstream actions of TGF-β or whether IL-13 can also exhibit direct profibrotic activity. The answers to these questions are important, particularly in diseases where Th2 responses persist (3, 4, 14–16), because they may have major impact on both the design and long term benefit of future clinical interventions based on disrupting the TGF-β signaling pathway (17–19). In this study, we report a series of in depth studies designed to test the hypothesis that TGF-β1 is required for the development of IL-13-dependent fibrosis in schistosomiasis. Importantly, these studies provide solid evidence of an IL-13-driven pathway of fibrogenesis that is completely MMP-9, Smad3, and TGF-β independent.

Materials and Methods

Mice, infections, and parasite materials

Six- to 8-wk-old female C57BL/6 and 129 SvEv mice were obtained from Taconic Farms (Germantown, NY). Breeding pairs of IL-13$^{-/-}$, IL-4/13$^{-/-}$, and wild-type (WT) mice were maintained on 129Ola $\times$ C57BL/6 (F_2) background and were originally provided by Dr. A. McKenzie (Medical Research Council Laboratory, Cambridge, U.K.; Refs. 20 and 21). Tg mice expressing a TGF-β antagonist of the soluble type II TGF-β-Fc fusion class (SR2F), Smad3$^{-/-}$, TGF-β1$^{-/-}$mice, and their respective WT littermates were all bred at the National Institutes of Health (Bethesda, MD; Refs. 22–24). MMP-9$^{-/-}$ mice were bred on the 129SvEv background and129SvEv controls were purchased from Taconic Farms. The MMP-9$^{-/-}$ animals were kindly provided by M. Shipley and R. Senior (Barnes-Jewish Hospital at Washington University, St. Louis, MO; Ref. 25). Age- and sex-matched mice were exposed percutaneously through the tail to 35 cercariae of a Puerto Rican strain of *Schistosoma mansoni* (Naval Medical Research Institute, Bethesda, MD) obtained from infected *Biomphalaria glabrata* snails (Biomedical Research Institute, Rockville, MD). Soluble *S. mansoni* egg Ag (SEA) was purified from homogenized eggs as previously described (26). The murine TBRII-Fc chimera was a kind gift from P. Gotwals (Biogen, San Diego, CA). For the TBRII-Fc studies, BALB/c mice were infected with 50 cercariae by s.c. injection. Starting at day 52 postinfection (p.i.), mice were administered 100 μg of chimera in PBS by i.p. injection once per day. Controls received PBS alone. Mice were euthanized on day 62 p.i.. Anti-TGF-β Ab (specific for the PAN region of TGF; specifically blocks TGF-β1 and -β2, with lesser neutralizing activity for TGF-β3; clone 2G7, originally from Genentech, South San Francisco, CA; Ref. 27) An isotype-matched anti-βgal Ab was used as a control. All mice were treated three times weekly with 200 μg per mouse per day starting on wk 5 p.i. and ending on wk 9 at the time of sacrifice. In other studies, TGF-β1$^{-/-}$ mice and their WT littermates were injected with 10 μg of rIL-13 in 200 μl of PBS and sacrificed after 24 h. rIL-13 was provided by Wyeth-Genetics Institute (Cambridge, MA). All animal studies were approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board (Bethesda, MD).

Histopathology and hydroxyproline assay

The size of hepatic granulomas around eggs containing live, mature embryos was determined in histologic sections stained by Wright's Giemsa stain (Histopath of America, Clinton, MD). The percentages of eosinophils, mast cells, and other cell types were evaluated in the same Giemsa-stained sections. For all measurements, 30 granulomas were examined, and the average value for each animal is reported. The number of schistosome eggs in the liver and gut, and the collagen content of the liver determined as hydroxyproline, were measured as described (26). Specifically, hepatic collagen was measured as hydroxyproline by the technique of Bergman and Loxley after hydrolysis of a 200-mg portion of liver in 5 ml of 6 N HCL at 110°C for 18 h. The increase in hepatic hydroxyproline was positively related to egg numbers in all experiments, and hepatic collagen is reported as the increase above normal liver collagen in micromoles per 10,000 eggs or per worm pair: (infected liver collagen $-$ normal liver collagen)/liver eggs $\times$ 10^4 or micromoles per worm pair. In some studies, tissue slides were stained with trichrome to show areas of collagen deposition. The same individual scored all histologic features and had no knowledge of the experimental design.

Real-time PCR

Liver tissues were homogenized in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) using a tissue polytron (Omni International, Warrenton, VA), and total RNA was extracted following the recommendations of the manufacturer. RNA was further purified using the RNA Cleanup procedure with the RNeasy kits (Qiagen, Valencia, CA). A RT-PCR procedure was used to determine relative quantities of mRNA for several cytokine genes after reverse transcription of 1 μg of RNA (28). The cDNA was run on ABI PRISM 7900 Sequence Detection System in the SYBR Green PCR Master Mix (ABI P/N 4309155; Applied Biosystems, Foster City, CA). The amount of PCR product was determined by the comparative C_t method as described by Applied Biosystems for the ABI Prism 7700/7900 Sequence Detection Systems, normalized to hypoxanthine-guanine phosphoribosyl transferase and expressed as a fold increase or decrease compared with uninfected controls.

Cell culture and cytokine assays

For in vitro cytokine measurements, mesenteric lymph nodes (MLN) and spleens were removed aseptically 8 wk after infection, and single cell suspensions were prepared. Mesenteric nodes were assayed from each mouse individually and cells were plated in 24-well tissue culture plates at a final concentration of 3 $\times$ 10^6 cells/ml in RPMI 1640 supplemented with 2 mmol/L glutamine, 25 mmol/L HEPES, 10% FCS, 50 μmol/L 2-ME penicillin, and streptomycin. Spleens were pooled from 5 to 10 animals per group, and cells were plated at a final concentration of 4 $\times$ 10^6 cells per milliliter using the same medium. Cells from the granulomatous livers were isolated as described in detail elsewhere (29). Cultures were incubated at 37°C in 5% CO_2. MLN cells and splenocytes were stimulated with SEA (20 μg/ml), soluble worm Ag preparation (SWAP; 50 μg/ml), or medium alone. Supernatant fluids were harvested at 72 h and assayed for cytokine activity. IL-13 and TGF-β levels were measured using capture ELISA kits supplied by R&D Systems (Minneapolis, MN), following manufacturer's instructions. Cytokine levels were tested in duplicate, and standard curves were generated using recombinant murine cytokines.

Statistics

Hepatic fibrosis (adjusted for egg number) decreases with increasing intensity of infection (worm pairs). Therefore, these variables were compared by analysis of covariance, using the log of total liver eggs as the covariate and the log of hydroxyproline per egg. Variables that did not change with infection intensity were compared by one-way ANOVA or by Student's *t* test. Changes in cytokine mRNA expression and granuloma size were evaluated by ANOVA. Results were considered significant where $p < 0.05$.

Results

In the absence of IL-13 fibrogenesis is reduced but TGF-β production is unimpaired following infection with S. mansoni

To determine whether there is a link between IL-13 and TGF-β1 in the pathogenesis of schistosomiasis-induced liver fibrosis, we first examined whether production of TGF-β1 is strictly dependent on IL-13 following infection (6, 30). For these studies, WT, IL-13$^{-/-}$, and IL-4$^{-/-}$/IL-13$^{-/-}$ double-deficient mice were infected percutaneously with *S. mansoni* cercariae. The animals were sacrificed on wk 8 p.i., the point where the egg-induced inflammatory response reaches a peak, and examined for several parasitological and immunological features. All mice harbored similar numbers of worm pairs and tissue egg burdens did not vary, confirming that the establishment of infection was comparable between the three

groups (data not shown). However, fibrosis measured by the hydroxyproline assay was significantly elevated in WT mice compared with the IL-13$^{-/-}$ and IL-4$^{-/-}$/IL-13$^{-/-}$ mice (Fig. 1*A*). Interestingly, the decrease in fibrosis in the IL-13$^{-/-}$ and IL-4$^{-/-}$/IL-13$^{-/-}$ mice was the same, confirming the strict requirement for IL-13 (3, 5) in this process. In contrast, granuloma size decreased significantly only in the double-deficient mice (Fig. 1*A*, *inset*), confirming nonredundant roles for IL-4 and IL-13 in the egg-induced granulomatous response (5).

To determine whether the decrease in fibrosis in the IL-13$^{-/-}$ and IL-4$^{-/-}$/IL-13$^{-/-}$ mice was associated with a reduction in TGF-β1 production, mRNA was isolated from granulomatous liver tissue at 8 wk p.i. and real-time RT-PCR performed for IL-13, IL-4, and TGF-β. WT mice showed a marked up-regulation in IL-13 (>200-fold) and a more modest increase in IL-4 (20-fold) and TGF-β (2-fold) following infection (Fig. 1*B*). The two knockout (KO) strains were deficient in IL-13 or both IL-4 and IL-13, as expected. However, more importantly, the level of TGF-β1 was the same in all groups, suggesting that in this model, IL-13 and IL-4 are not required for the up-regulation of TGF-β1 mRNA (Fig. 1*B*). Next, to determine whether the levels of the TGF-β protein were consistent with the mRNA results, MLN, spleens, and livers were isolated from infected mice and single-cell suspensions prepared. Lymphocyte cultures were restimulated in vitro in the presence of SEA and SWAP for 72 h. Culture supernatants were analyzed by ELISA for TGF-β1 (Fig. 1*C*). The spleen and LN cultures from all three infected groups produced TGF-β1 constitutively. However, more importantly, consistent with the liver mRNA results, there was no evidence of a reduced TGF-β1 response in either of the KO groups. In fact, there was slightly more SEA-induced TGF-β1 in the granuloma cultures prepared from IL-13$^{-/-}$ mice, not to mention a 2-fold increase in IL-13$^{-/-}$ SWAP-stimulated lymph nodes. Thus, even though liver fibrogenesis decreases substantially in the IL-13$^{-/-}$ and IL-4$^{-/-}$/IL-13$^{-/-}$ mice, this is not accompanied by a significant change in TGF-β1 production.

MMP-9 is not required for IL-13-dependent fibrosis during chronic S. mansoni *infection*

TGF-β is produced in a latent form; the TGF-β dimer being bound to its propeptide, latency-associated peptide. MMP-9 can proteolytically cleave latent TGF-β, transforming it to its active form (31). Interestingly, it was recently shown that MMP-9 mRNA is markedly up-regulated in the lungs of CC-10-IL-13 Tg mice. Moreover, when a MMP-9 null mutation was introduced into the IL-13 Tg mice, there was a significant decrease in the levels of spontaneously active TGF-β1 in bronchoalveolar lavage fluids (6). Thus, the investigators suggested that MMP-9 plays a critical role in IL-13-induced TGF-β activation and fibrosis. To determine whether a similar mechanism is operating in IL-13-induced liver fibrosis, WT C57BL/6 and MMP-9$^{-/-}$ mice were sacrificed at

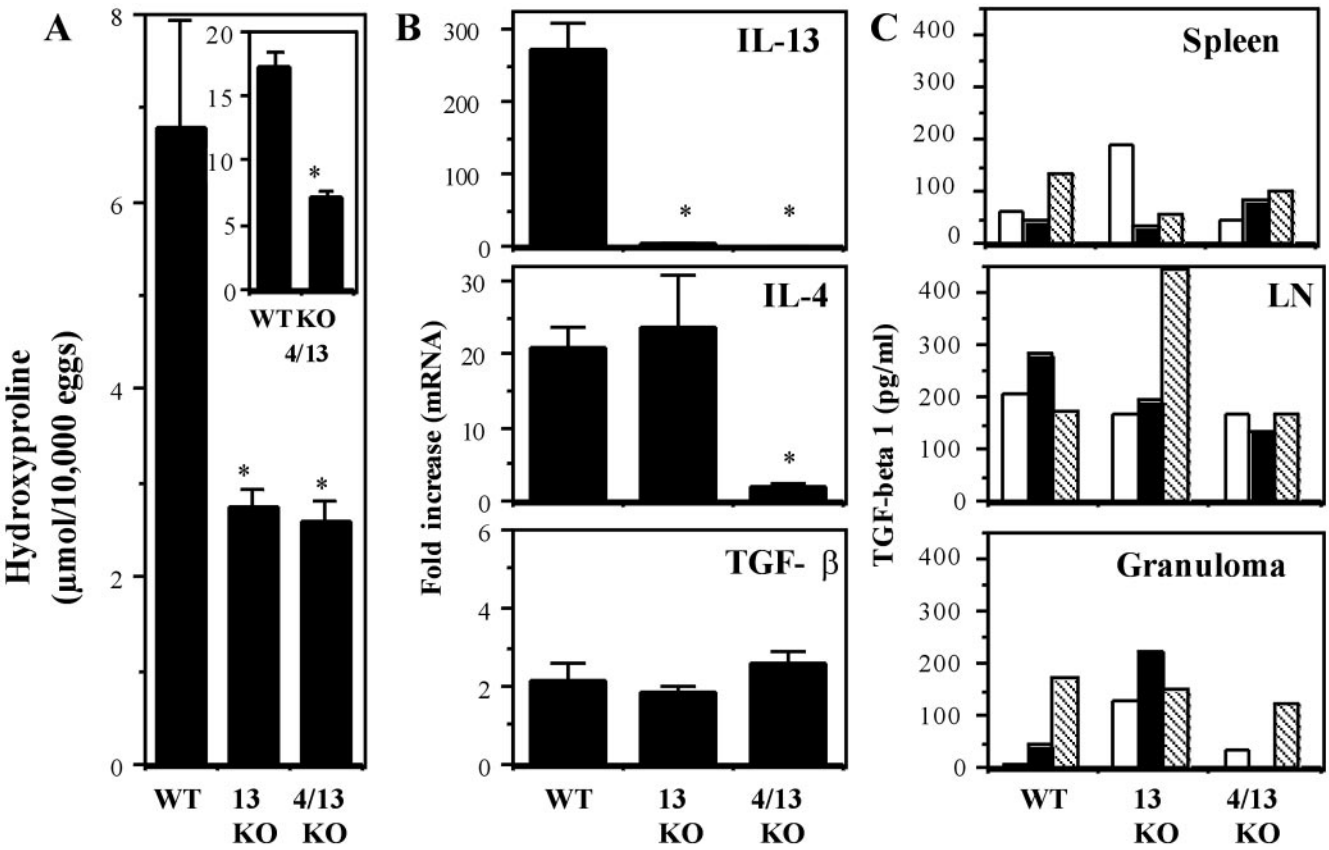

FIGURE 1. In the absence of IL-13, liver fibrosis decreases but production of TGF-β is unimpaired. Groups of WT (n = 10), IL-13$^{-/-}$ (n = 8), and IL-4/IL-13$^{-/-}$ (n = 16) mice were infected with 35 *S. mansoni* cercariae. All animals were sacrificed at 8 wk p.i. *A*, Fibrosis was assessed by the amount of hydroxyproline in micromoles detected in the liver per 10,000 eggs ± SE. The small *inset* in *A* shows the average granuloma volume for WT and IL-4/IL-13$^{-/-}$ mice measured in mm^3 × 10^{-3}. *B*, RNA isolated from individual liver specimens was analyzed by real-time PCR for IL-13, IL-4, and TGF-β mRNA, and expressed as a fold-increase over uninfected WT control. *C*, TGF-β production was analyzed in the spleen, MLN, and granuloma-associated lymphocytes after a 72-h incubation in the presence of medium alone (open bars), SEA (20 μg/ml, filled bars), or SWAP (50 μg/ml, hatched bars) and reported as means ± SD. *, Significant difference when compared with WT controls.

acute (wk 8) and chronic (wk 12) time points after infection (32). We found no difference between the WT and MMP-9$^{-/-}$ mice in granuloma size or tissue eosinophilia at either time point (Fig. 2*A*). More importantly, the MMP-9$^{-/-}$ mice also showed no impairment in fibrogenesis. Between wk 8 and 12 p.i., WT and MMP-9$^{-/-}$ mice showed comparable increases in fibrosis (Fig. 2*A*, *bottom panel*). The levels of IL-13, IL-4, and TGF-β mRNA were also quantified in the liver by real-time PCR and similarly increased in both groups (Fig. 2*B*). Therefore, the absence of MMP-9 had no significant effect on the generation of the major profibrotic cytokines. Protein levels of IL-13 and TGF-β were also determined by ELISA using splenocyte (Fig. 2*C*) and MLN cultures (data not shown). In agreement with the liver mRNA results, the levels of TFG-β and IL-13 were similar in the WT and MMP-9$^{-/-}$ mice. Although production of TGF-β and IL-13 was slightly increased in the MMP-9$^{-/-}$ mice, this was not significant nor was it observed in every experiment. To further confirm that IL-13 was produced at similar levels in both groups, concentrations of IL-13 were also examined in the serum and found to be at nearly identical levels (Fig. 2*C*, *inset, bottom panel*). Thus, although MMP-9 was shown to be critical in the IL-13 Tg mouse studies (6), our results suggest that MMP-9 is dispensable in the development of IL-13-dependent liver fibrosis. Because MMP-9 is not the only activator of latent TGF-β, it also remains possible that other activators, such as MMP-2 (31), could be playing a role in the activation of TGF-β. Alternatively, these data may suggest that TGF-β1 is not required for the generation of fibrosis in this model.

TGF-β1 blockade fails to regulate the development of IL-13-dependent liver fibrosis

To examine more directly the requirement for TGF-β, in subsequent experiments, we used a variety of KO/Tg mice, Abs, and inhibitors that disrupt all or part of the TGF-β1, 2, and 3 signaling cascade. Although it would have been ideal to conduct infection studies with TGF-β1 null mice, deletion of the TGF-β1 gene results in the death of 60% of mouse embryos, with the remainder exhibiting a multifocal inflammatory response within a few weeks of birth, thus making such studies impossible. Instead, we used several approaches to specifically inhibit TGF-β activity after infection. In our first strategy, WT C57BL/6 mice were infected and at 5 wk p.i. and separate groups were treated with a neutralizing anti-TGF-β Ab, control Ig, or nothing as described in *Materials and Methods*. The neutralizing mAb is against the TGF-β PAN region, which effectively blocks both TGF-β1 and -β2 (27). All mice were sacrificed on wk 8 p.i. Here again, no changes in the establishment of infection were noted because the animals harbored similar worm and tissue egg burdens (data not shown). The egg-induced inflammatory response also appeared normal in all three groups because granuloma size and the percentage of tissue eosinophils were unchanged (Fig. 3*A*). Furthermore, neutralizing TGF-β had no effect on fibrosis as all groups of mice displayed similar liver hydroxyproline levels (Fig. 3*A*, *bottom panel*). Additional studies performed with mice on the high pathology C3H/HeN background (33, 34) produced similar results (data not

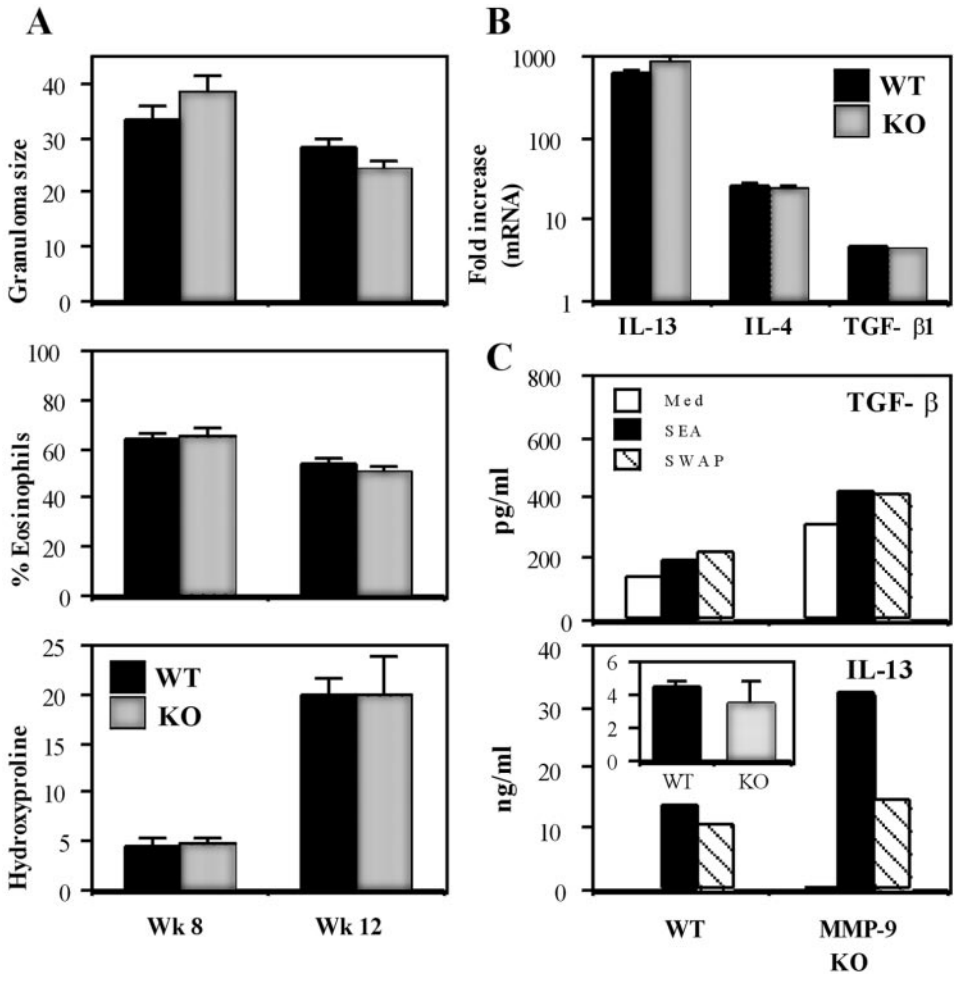

FIGURE 2. IL-13-dependent liver fibrosis is MMP-9-independent. *A*, WT (filled bars) and MMP-9$^{-/-}$ (shaded bars) mice were infected with 35 *S. mansoni* cercariae and sacrificed on wk 8 and 12 p.i. to determine average granuloma size, tissue eosinophilia, and hydroxyproline level (8–10 mice per group per time point). *B*, RNA isolated from individual liver specimens was analyzed by real-time PCR for IL-13, IL-4, and TGF- β1 mRNA and expressed as a fold-increase over uninfected WT control. *C*, TGF-β and IL-13 production was analyzed in pooled splenocyte cultures ($n = 5$) prepared from 8-wk infected WT and MMP-9$^{-/-}$ mice. The small *inset* in *C* (IL-13) shows the average serum levels of IL-13 in each group of mice at 8 wk p.i. $\pm$ SD ($n = 5$). Background levels of IL-13 were below 1 ng/ml for each group (data not shown).

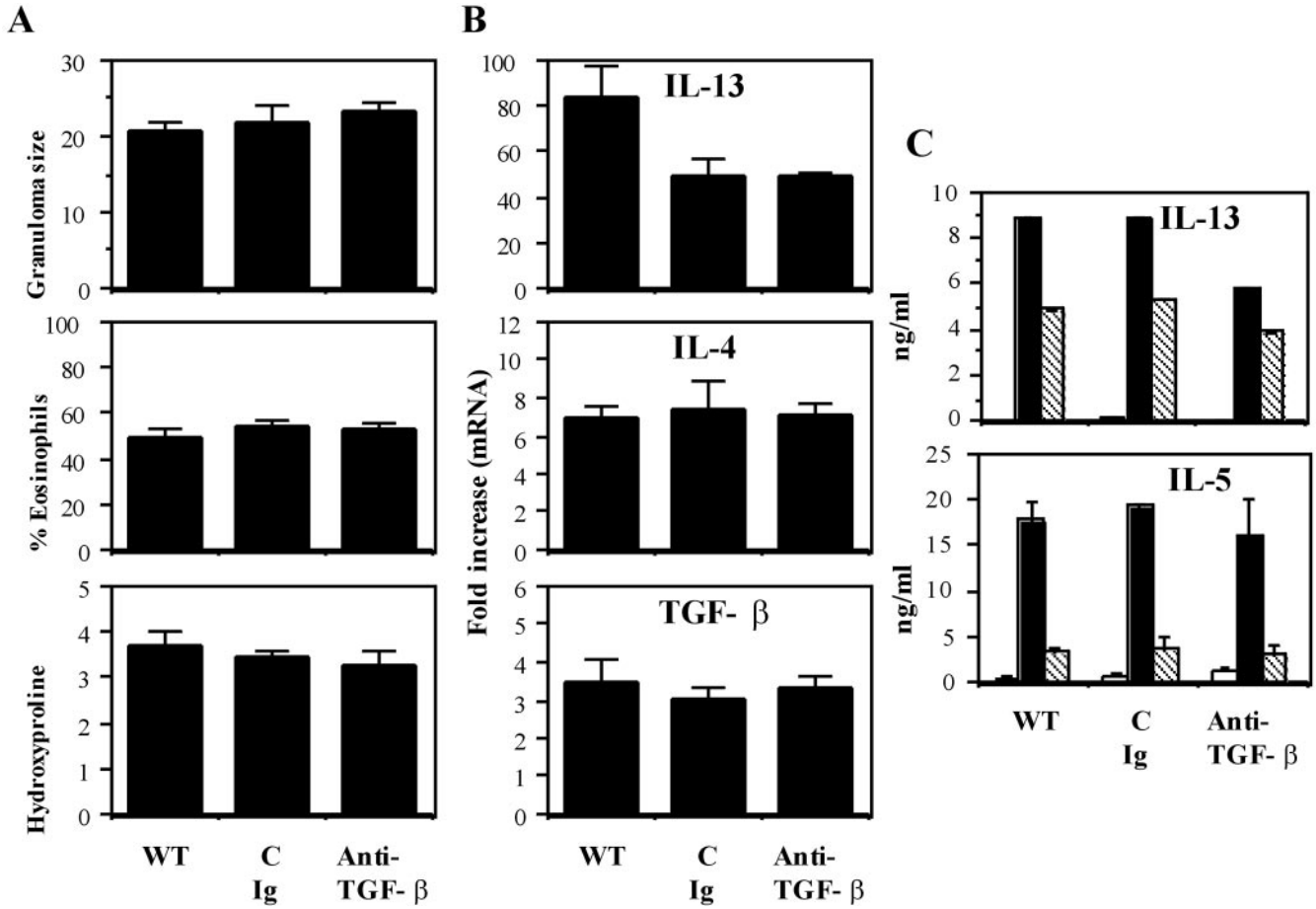

FIGURE 3. Liver pathology induced by *S. mansoni* infection is unchanged by anti-TGF-β mAb treatment. *A*, WT C57BL/6 mice were infected with 35 *S. mansoni* cercariae and treated with control Ig (GL113) or anti-TGF-β mAb (2G7) from wk 5 to 8 (200 μg/mouse, three times weekly) p.i. All mice were sacrificed on wk 8 to determine average granuloma size, tissue eosinophilia, and hydroxyproline level ($n = 8$ per group). *B*, RNA isolated from individual liver specimens was analyzed by real-time PCR for IL-13, IL-4, and TGF-β mRNA and expressed as a fold-increase over uninfected WT control. *C*, IL-13 and IL-5 production was analyzed in pooled splenocyte cultures ($n = 5$) after a 72-h incubation in medium alone (open bars), SEA (20 μg/ml, filled bars), or SWAP (50 μg/ml, hatched bars).

shown). There was also no significant effect on the local cytokine response, with IL-13, IL-4, and TGF-β mRNA levels remaining similarly elevated in the three groups (Fig. 3*B*). Consistent with these observations, protein levels of the Th2-associated cytokines IL-13 and IL-5 were also similar (Fig. 3*C*). This was also true using MLN cultures (data not shown).

In a second approach to neutralize TGF-β activity, we used Tg mice that over-express a TGF-β antagonist of the soluble TGF-βRII-Fc fusion protein class (24). These Tg mice express biologically significant levels of antagonist in the serum and in most tissues, and previous studies suggested the soluble receptor selectively neutralizes undesirable TGF-β, while not affecting TGF-β involved in the maintenance of normal homeostasis. To determine whether over-expression of the antagonist would regulate liver fibrosis following schistosome infection, WT and Tg mice were infected. TGF-βRII-Fc Tg mice were compared with their WT littermates at 8 wk p.i.. Interestingly, there was a small but consistent decrease in peak granuloma size in the Tg-positive animals (~25%; Fig. 4). Nevertheless, this was not accompanied by any change in fibrosis or in the number of granuloma-associated eosinophils, suggesting that the animals developed a normal type-2-polarized immune response, which was confirmed by analyzing liver tissues by real-time PCR (data not shown).

In a third approach, infected WT mice were treated aggressively with a soluble chimeric TGF-βRII-Fc protein. This protein is highly effective at inhibiting TGF-β and has been used successfully in a variety of clinically relevant models (35, 36). Lee et al. (6) showed that when CC-10-IL-13 Tg mice are injected with the chimeric fusion protein, collagen deposition in the lung is markedly reduced, thereby reversing the pathology associated with sustained IL-13 production. Nevertheless, when a similar strategy was used in the schistosomiasis model, we observed no change in collagen deposition, suggesting that other mediators besides TGF-β are playing more important roles (Fig. 5). When viewed together, the results from all of these approaches strongly suggest that TGF-β1 is dispensable for the development of IL-13-driven fibrosis in schistosomiasis, thus arguing against a linear pathway in which IL-13 must induce and activate TGF-β1 to trigger the fibrogenic machinery (6).

The Smad3 signaling molecule has no effect on IL-13 production and is dispensable for IL-13-driven liver fibrosis

Because it remains possible that another isoform of TGF-β is involved, in a final strategy, we used mice in which TGF-β signaling is compromised. As explained above, Smad proteins are responsible for the downstream signaling that results when active TGF-β binds its receptor. Among the Smad proteins involved in TGF-β signaling, only Smad 2 and Smad 3 have been disrupted in mice. However, disruption of Smad 2 is not compatible with normal

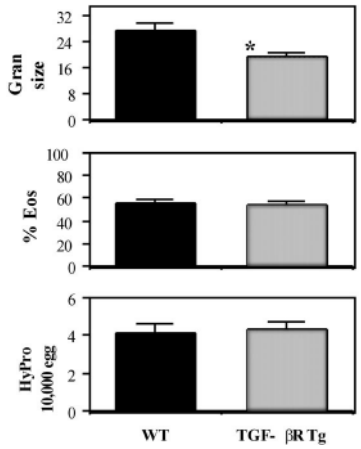

FIGURE 4. TGF-β antagonist mice show no decrease in liver fibrosis following infection with *S. mansoni*. *A*, WT (filled bars) and soluble TGF-βRII-Fc mice (shaded bars) mice were infected with 35 *S. mansoni* cercariae and sacrificed on wk 8 (n = WT, 9 and KO, 8) to determine average granuloma size, tissue eosinophilia, and hydroxyproline level. *, Significant difference when compared with WT controls ($p = 0.039$).

development (37–39). Therefore, for these studies, Smad3-deficient mice were used (40, 41) and infected with *S. mansoni* as described in previous experiments. As seen in the MMP-9$^{-/-}$ and TGF-β-blocking studies, granuloma formation and tissue eosinophilia were unchanged in the Smad3 null mice at both acute and chronic time points p.i., with normal down-modulation of granuloma size observed by wk 12 (Fig. 6*A*). Liver hydroxyproline levels also increased normally during this period (Fig. 6*A*, *bottom panel*). To determine whether Smad3 had any effect on the production of IL-13, splenocytes were stimulated in vitro with *S. mansoni* Ag and culture supernatants were tested for IL-13 by ELISA. Importantly, no change in IL-13 production was seen, which likely explains their unaltered pattern of liver fibrogenesis (Fig. 6*B*). Similar results were also obtained from MLN cultures and real-time PCR analysis of liver mRNA (data not shown). Thus, although it has been previously shown that Smad3-deficiency ameliorates all fibrotic sequelae in which TGF-β has been implicated (23, 42–44), liver fibrosis induced by chronic *S. mansoni* infection progresses normally in the absence of this down-stream TGF-β signaling molecule. Because Smad3 is involved in the induction of TGF-β through an autocrine loop, Smad3 null mice also produce lower amounts of TGF-β (23), further diminishing the possibility that residual TGF-β signaling (from any isoform) is playing an important role.

Several genes involved in tissue remodeling and repair are induced directly by IL-13 in TGF-β1 null mice: evidence of a TGF-β1-independent mechanism of fibrosis

To determine whether genes involved in wound healing and fibrosis can be activated by IL-13 in the absence of TGF-β1, TGF-β1 null mice were treated with recombinant murine IL-13 (rMuIL-13). At various time points after injection of a single dose of cytokine, animals were sacrificed, liver RNA was isolated, and real-time PCR was performed to determine whether several genes involved in ECM remodeling were up-regulated in both WT and TGF-β1$^{-/-}$ mice. Because TGF-β1$^{-/-}$ mice survive only 4–8 wk after birth (45), KO and WT littermates were used in all experiments at ~2 wk of age. In initial studies, mice were injected with rMuIL-13 or saline and sacrificed 24 h later. mRNA levels were expressed as a fold change in gene expression after challenge using naive TGF-β1$^{-/-}$ mice as the baseline so that differences between naive WT and TGF-β1$^{-/-}$ mice could also be easily discerned.

As expected, there was no detectable TGF-β1 message in any of the TGF-β1$^{-/-}$ mice with or without rMuIL-13 (Fig. 7). In agreement with other studies (6, 46), we also found a significant 3-fold induction in TGF-β1 mRNA expression in WT mice after stimulation with rMuIL-13. To determine whether collagen gene expression could be activated by IL-13 in the presence or absence of TGF-β, expression of the interstitial collagens was also examined, including Col I and Col III. Strikingly, a 20- to 50-fold induction over the background was observed in the TGF-β1$^{-/-}$ mice after IL-13 treatment (Fig. 7). Although the IL-13-treated WT group showed a slightly stronger response, the fold-changes in Col I and Col III induced by IL-13 were remarkably similar for both groups when their respective background levels were taken into consideration. This was due to the relatively high basal levels of Col I and Col III mRNA observed in the untreated WT mice. Collagen XIV showed a similar response (data not shown). Thus, although TGF-β1 influences the background levels of the interstitial collagens, the ability of exogenous IL-13 to induce collagen gene expression in vivo was normal in the absence of TGF-β1.

Regulation of several other ECM-related genes was also examined in these experiments, including several MMPs and their inhibitors, the tissue inhibitors of metalloprotease (TIMPs). MMPs are a family of zinc-and calcium-dependent proteases that together

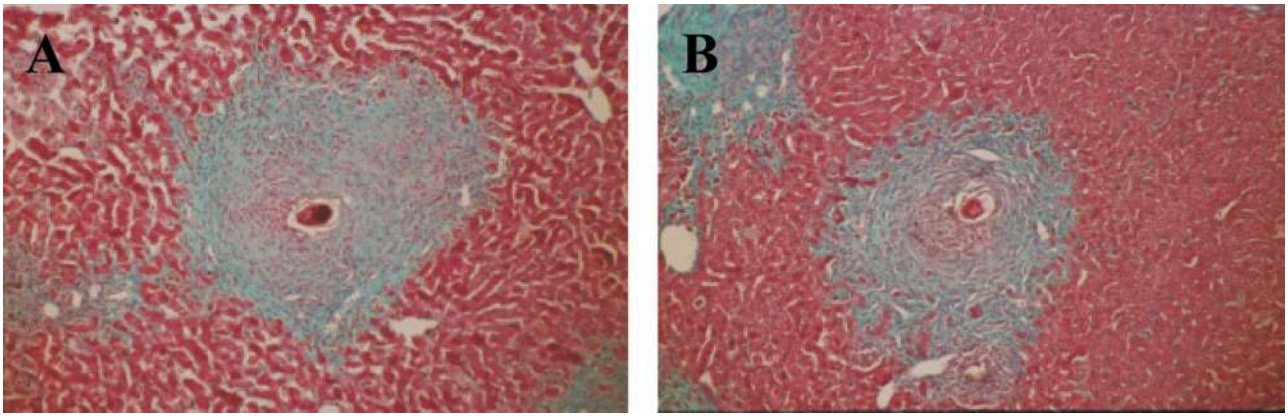

FIGURE 5. Fibrosis develops normally in mice treated with the soluble TGF-βRII-Fc antagonist. *S. mansoni* infected WT (*A*) and WT mice treated with chimeric TGF-βRII-Fc (*B*) were sacrificed on wk 9 p.i. Liver sections were stained with Trichrome (blue) to stain collagen and a representative granuloma is shown (magnification, ×200). This experiment was repeated one time with similar results.

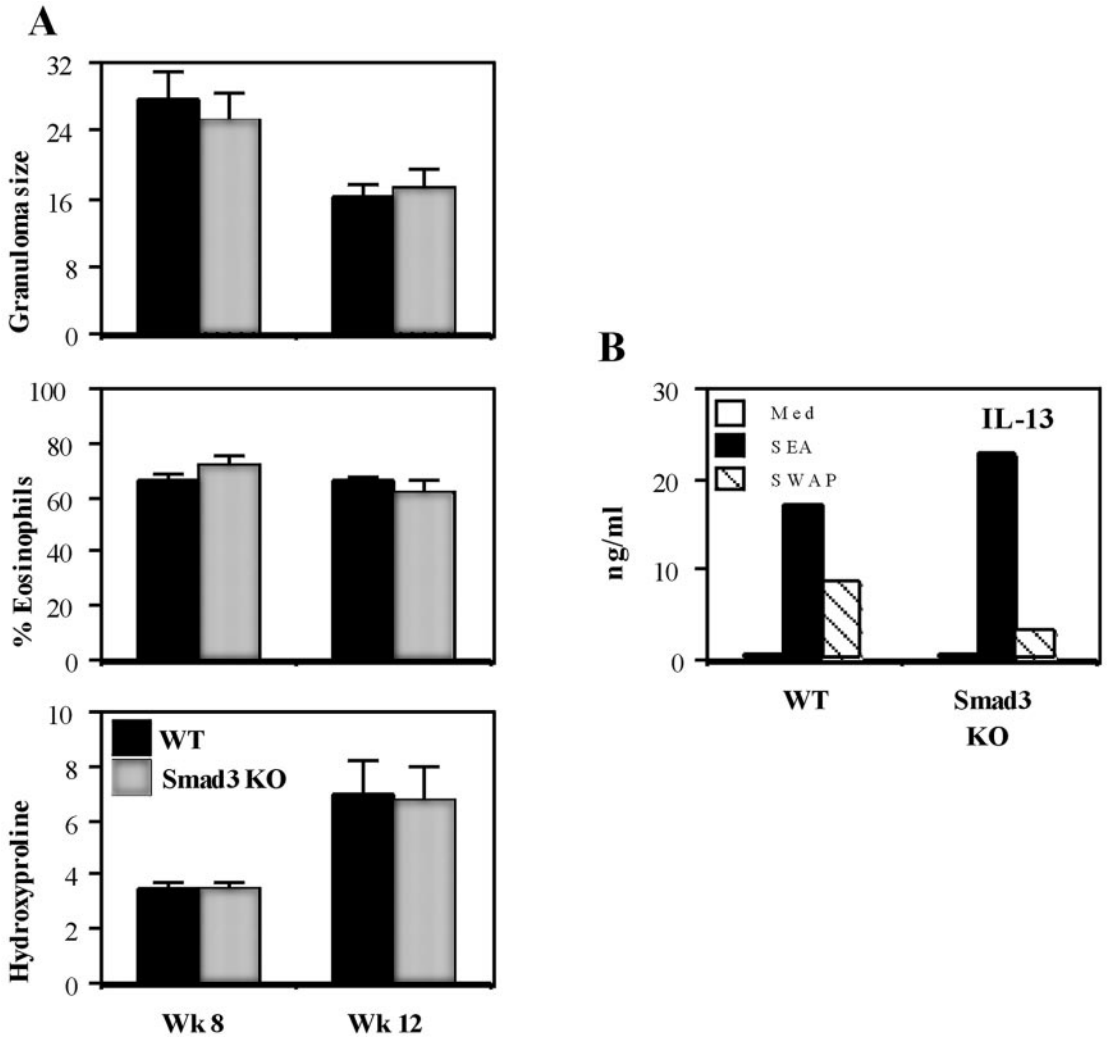

FIGURE 6. Development of liver fibrosis and production of IL-13 does not require the Smad3 signaling molecule. *A*, WT (filled bars) and Smad3$^{-/-}$ (shaded bars) mice were infected with 35 *S. mansoni* cercariae and sacrificed on wks 8 (n = WT, 17 and KO, 15) and 12 p.i. (n = WT, 16 and KO, 10) to determine average granuloma size, tissue eosinophilia, and hydroxyproline level. *B*, IL-13 production was analyzed in pooled splenocyte cultures (n = 5) prepared from 8-wk infected WT and Smad3$^{-/-}$ mice as described in the legend to Fig. 1. The results shown are the combined data from two independent experiments.

degrade virtually all components of the ECM. It has been suggested that the TIMPs, via their MMP inhibitory activity, are important for the progression of fibrosis by regulating the turnover of the ECM. Thus, fibrotic diseases including schistosomiasis are almost always associated with significant changes in MMP and TIMP gene expression (47). As discussed above, MMP-9 and MMP-2 have both been implicated in the activation of latent TGF-β. Consistent with the Col I and Col III findings, mRNA for both of these genes markedly increased in the liver in response to IL-13 and there was no evidence that expression was dependent on TGF-β. However, there was a consistent decrease in MMP-3 expression in the TGF-β1$^{-/-}$ mice, suggesting that expression of some members of the metalloproteinase family may be dependent on TGF-β. However, interestingly, there were additional genes, including MMP-13 and to a lesser extent MMP-9, where IL-13 actually increased gene expression even more in the absence of TGF-β. Moreover, TIMP-1, considered a useful diagnostic marker of hepatic fibrosis (48) and the most ubiquitous of all of the TIMPs, followed a similar TGF-β-independent pattern. Additional fibrosis-related genes were also examined including fibronectin (not shown), fibrillin, and tenascin (Fig. 7). Although studies have shown that fibronectin and tenascin are up-regulated by TGF-β1, our findings suggest that all three genes can also be significantly induced by IL-13 in the presence or absence of TGF-β1 (49). Microarray experiments performed with oligonucleotide chips containing ~17,000 mouse genes confirmed many of these findings (results not shown).

Discussion

Perhaps because of its wide-ranging effects in the body, TGF-β is released from cells in a latent form, and its activation and downstream signaling are complex, involving a variety of mediators. Thus, it appears extremely important to fine-tune its activity in vivo (8). Although, manipulating TGF-β by inhibiting its expression, activation, or signaling has been used successfully to reduce tissue scarring and fibrosis in some animal models, to date, few of these approaches have reached a clinical trial (50). Given the wide-ranging interest in developing therapeutics based on targeting TGF-β, understanding the upstream and downstream mechanisms that regulate its activity is of critical importance. The recent discovery that IL-13 is a potent inducer and activator of TGF-β was important (6) because it provided a possible explanation for the marked fibrotic tissue remodeling that accompanies chronic type-2 cytokine-polarized immune responses (7, 51, 52). Nevertheless,

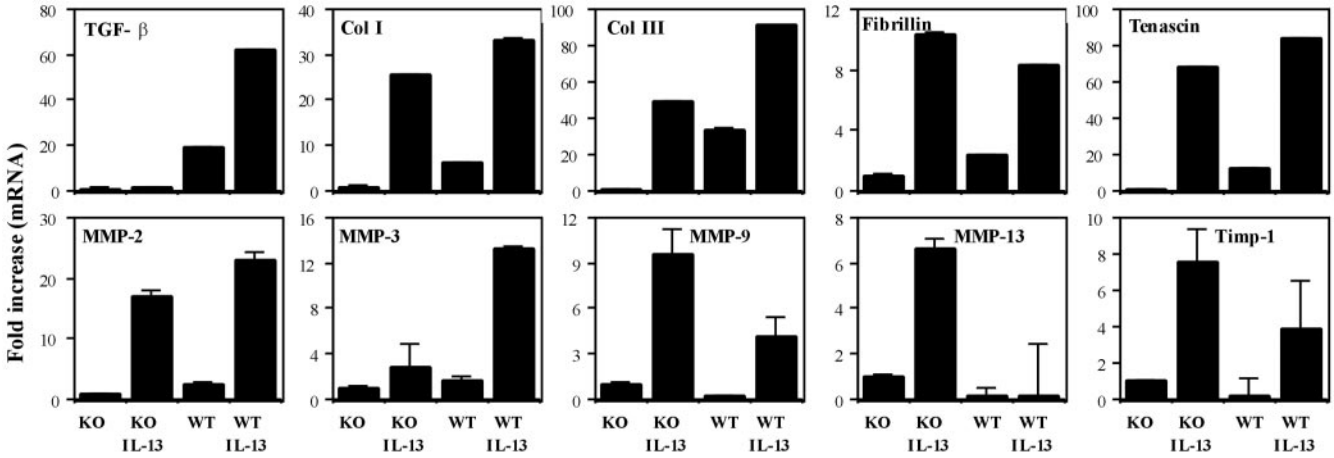

FIGURE 7. Several genes involved in tissue remodeling and fibrosis are induced by rIL-13 and are TGF-β independent. Two-week-old TGF-β1 null (KO) and their WT littermates (WT) were injected with rIL-13 (KO IL-13 and WT IL-13) or saline and sacrificed after 24 h. mRNA was extracted from the livers of mice (samples from the different mouse groups were pooled) and real-time PCR was performed as described in *Materials and Methods* for several ECM remodeling genes. Similar results were obtained in two repeat experiments.

the relative importance of this step-wise mechanism of fibrosis (IL-13 $\rightarrow$ TGF-β $\rightarrow$ fibrosis) was never fully investigated in a model system in which fibrosis develops as a natural sequela to chronic infection, or following exposure to a persistent inflammatory stimulus. Strikingly, the combined findings from our studies of schistosomiasis-induced liver fibrosis argue against an IL-13-mediated mechanism of fibrosis that is strictly dependent on TGF-β. Thus, targeting TGF-β alone may prove inadequate, particularly in conditions where Th2-cytokines like IL-13 have been implicated in the pathological tissue remodeling (7, 51, 52).

The results from these experiments were surprising because previous studies, in addition to our own, found significant increases in TGF-β expression following schistosome infection implicating it in the etiology of the disease (53–55). Nevertheless, the findings reported in these earlier studies were only correlative, with none investigating the contribution of TGF-β by any direct functional assay (56–59). We initially examined whether production of TGF-β was dependent on IL-13, because this hypothesis was inferred by the IL-13 Tg mouse studies (6). Although the studies we conducted with rIL-13 confirmed a role for IL-13 in the induction of TGF-β1 expression, we found no decrease in TGF-β1 in infected IL-13$^{-/-}$ or IL-4$^{-/-}$/IL-13$^{-/-}$ mice either locally and systemically, suggesting that Stat-6-dependent signaling pathways are not strictly required. Because TGF-β production appeared normal in the absence of IL-13, yet liver fibrosis decreased substantially, this was one of the first observations questioning the contribution of TGF-β. However, functional studies were required to formally invalidate its role. To investigate this further, we used an exhaustive number of strategies to disrupt all or part of the TGF-β signaling cascade. Importantly, with all approaches we failed to see any significant reduction in fibrosis. This was also true at chronic time points p.i., thereby eliminating a significant involvement of TGF-β in the progression of the disease.

In agreement with the IL-13 Tg mouse, we also observed a marked up-regulation in MMP-9 expression and related studies in the schistosomiasis model suggested that the egg-induced Th2 response was driving expression of several MMP genes (47, 60). However, consistent with the TGF-β blocking studies, no impairment in IL-13 production or in the development of hepatic fibrosis was observed in MMP-9-deficient animals, eliminating any requirement for MMP-9-induced TGF-β activation in this model. Why our findings differ so significantly from the CC10-IL-13 Tg mouse studies remains unclear, although multiple explanations could be involved. The most simple and perhaps obvious explanation is that IL-13 is made at significantly greater levels than TGF-β in schistosomiasis. Indeed, while TGF-β was produced in low picogram amounts following infection, production of IL-13 routinely exceeded 10 ng/ml in cultured lymphocytes, indicating an ~100-fold difference between the two mediators. Because we and others have shown that IL-13 and TGF-β can both bind to fibroblasts and stimulate collagen deposition (3, 5, 61), in situations where IL-13 is over-produced, the relative contribution of TGF-β in fibrogenesis may be substantially diminished. IL-13 is also a potent inducer of arginase expression in both macrophages and fibroblasts (62, 63), which stimulates production of proline, the major building block of collagen (29, 64). Thus, in some circumstances, IL-13, like TGF-β, may act directly on important target cells and activate the fibrotic pathway. Alternatively, there may be subtle differences in the mechanisms that control fibrosis in different tissues. Our studies focused on liver fibrosis, so it remains possible that distinct pathways are operating in the lung vs liver. Similar comparative studies conducted in chronic murine asthma models could help clarify this issue (65–68).

Although an infection study in TGF-β1$^{-/-}$ mice would have been the ideal experiment to investigate a link between IL-13 and TGF-β, the early mortality of these animals makes it extremely difficult to study the mechanisms of fibrogenesis because collagen accumulates slowly. Nevertheless, to prove that IL-13 can activate the fibrotic pathway directly in vivo, independent of TGF-β, we designed a short-term experiment in TGF-β1$^{-/-}$ mice. Rather than infecting the animals, we injected rIL-13 i.p. and examined whether several genes known to be involved in fibrosis were induced in the liver in the absence of TGF-β1. Strikingly, several genes, including the interstitial collagens, TIMP-1, fibrillin, tenascin, and several matrix metalloproteinases, were markedly induced by IL-13, with no significant impairment in the TGF-β1$^{-/-}$ mice. Thus, the cumulative results from these studies suggest that IL-13 and TGF-β can both be characterized as profibrotic mediators. Although, cooperation may be observed in some settings (6, 69), the findings described here suggest that a linear pathway where IL-13 must induce and activate TGF-β1 to trigger fibrosis is not an absolute requirement.

An important future area of research will be to understand why multiple upstream mediators are involved in the activation of the

fibrotic machinery. For us, it is intriguing to speculate that IL-13 might be an important initiator of an "adaptive" healing program that is induced only during an ongoing inflammatory response, while the TGF-β pathway of fibrosis may be a more "innate" mechanism of healing (70). The fact that IL-13 is produced by cells of the adaptive immune response (predominantly $CD4^+$ Th2 cells) while TGF-β is produced by all hemopoietic cell populations might support such a conclusion. IL-$13^{-/-}$ mice are also fertile and manifest no developmental problems until an immune response is provoked. In contrast, TGF-$\beta1^{-/-}$ mice are compromised during embryonic development and at birth, again arguing for an intrinsic role for TGF-β but more of a conditional requirement for IL-13. Interestingly, we observed lower basal levels of collagen I and collagen III mRNA in the livers of naive TGF-$\beta1^{-/-}$ mice, while similar fold-increases were observed in both WT and TGF-$\beta1^{-/-}$ mice following stimulation with rIL-13. In contrast, naive IL-$13^{-/-}$ mice displayed no reduction in background levels of Col I or Col III (4, 5). Thus, multiple lines of evidence suggest that the timing and magnitude of production of TGF-β and IL-13 likely influences their individual contributions to the development of fibrosis.

In summary, while the work described here does not dispute a role for TGF-β in fibrogenesis (58, 71–73), it suggests that IL-13-mediated fibrosis is not strictly dependent on the downstream actions of TGF-β1 as was recently suggested (6, 74). These new findings may have important clinical ramifications because they suggest neutralizing TGF-β alone may be ineffective for the treatment of fibrosis, particularly for diseases where a vigorous type-2 cytokine response is also present. Recent studies of both murine and human *S. mansoni* have linked fibrosis with low production of IFN-γ and IL-10 and with high levels of IL-13 (5, 75, 76). Several studies in mice have also confirmed an IL-13-, IL-4Rα-, and Stat6-dependent pathway is critical to the development of fibrosis (77–80). Therefore, in schistosomiasis and perhaps other Th2-driven inflammatory diseases, inhibiting IL-13 either alone or in combination with TGF-β might prove a more effective intervention to slow the progression of fibrotic tissue remodeling.

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