Induction of IL-13 Triggers TGF-β_1-Dependent Tissue Fibrosis in Chronic 2,4,6-Trinitrobenzene Sulfonic Acid Colitis

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To investigate the immunopathogenesis of inflammation-associated fibrosis, we analyzed the chronic colitis and late-developing fibrosis occurring in BALB/c mice administered weekly doses of intrarectal 2,4,6-trinitrobenzene sulfonic acid. We showed first in this model that an initial Th1 response involving IL-12p70 and IFN-γ subsides after 3 wk to be supplanted by an IL-23/IL-25 response beginning after 4–5 wk. This evolution is followed by gradually increasing production of IL-17 and cytokines ordinarily seen in a Th2 response, particularly IL-13, which reaches a plateau at 8–9 wk. In vitro stimulation studies suggest that this IL-13 production is dependent on IL-23 and IL-25, but not on IL-12p70. We then show that IL-13 production results in the induction of an IL-13R formerly thought to function only as a decoy receptor, IL-13Rα_2, and this receptor is critical to the production of TGF-β_1 and the onset of fibrosis. Thus, if IL-13 signaling through this receptor is blocked by administration of soluble IL-13Rα_2-Fc, or by administration of IL-13Rα_2-specific small interfering RNA, TGF-β_1 is not produced and fibrosis does not occur. These studies show that in chronic 2,4,6-trinitrobenzene sulfonic acid colitis, fibrosis is dependent on the development of an IL-13 response that acts through a novel cell surface-expressed IL-13R to induce TGF-β_1. A similar mechanism may obtain in certain forms of human inflammatory bowel disease. ***The Journal of Immunology,*** **2007, 178: 5859–5870.**

Chronic inflammatory diseases often result in extensive tissue fibrosis and its associated untoward side effects on organ function. This effect is seen quite clearly in the two major forms of inflammatory bowel disease (1–5). Thus, in Crohn's disease (CD),[2] one sees a transmural, granulomatous inflammation that frequently leads to mechanical obstruction of the intestinal lumen due to extensive local tissue fibrosis (1, 2). In addition, in ulcerative colitis, one sees a more superficial and ulcerative inflammation that nevertheless results in a stiff, fibrotic large bowel unable to carry out peristalsis (1, 2). Thus, in both forms of inflammatory bowel disease, a fibrotic reaction supervenes that becomes a dominant part of the pathologic picture.

In recent studies, we have shown that tissue fibrosis is, at least in part, dependent on IL-13-induced TGF-β_1 production initiated by IL-13 signaling through cell membrane-expressed IL-13Rα_2 (6, 7). The latter is a receptor previously thought to function only as a soluble IL-13 decoy but is now known to allow signaling of monocytes and macrophages. Such signaling results in activation of an AP-1 transcription family member composed of c-jun and Fra-2 and perhaps other transcription factors that act on the TGF-β_1 promoter to induce production of TGF-β_1 and subsequent initiation of downstream collagen production. Of interest, we have shown that this signaling mechanism is operative in the acute Th2 hapten-induced colitis known as oxazolone colitis, a model of human ulcerative colitis in which the production of TGF-β_1 is a prominent feature of the inflammation (6, 8).

For further studies of fibrosis occurring in a model of colitis, we turned to the hapten-induced colitis caused by an intrarectal administration of TNBS (2,4,6-trinitrobenzene sulfonic acid) and ethanol, known as TNBS colitis (9). When TNBS colitis is induced in SJL/J or C57BL/10 mice by a single relatively large dose of TNBS, it takes the form of an IL-12-driven, Th1-mediated transmural colonic inflammation that is dependent on the activation of the central inflammatory transcription factor NF-κB (10–12). In this setting, it is an acute colitis lasting 5–9 days and thus cannot be used to study fibrosis. In contrast, when induced by weekly administration of a relatively low dose of TNBS in BALB/c mice, TNBS colitis is a chronic colitis that is marked by colonic collagen deposition after the fifth week (10, 13). Given the association of fibrosis with IL-13 alluded to above, this process suggested that chronic TNBS colitis in BALB/c mice might have a unique cytokine profile.

In the present study, we showed that chronic TNBS colitis in BALB/c mice is initially characterized by the production of the Th1 cytokines IL-12 and IFN-γ, but after the third week of TNBS treatment, this process is supplanted by the production of IL-23 and a cytokine known to be dependent on the latter, IL-17; in addition, another IL-17 family member, IL-17E (IL-25), makes its appearance. Then, at about the fifth week of TNBS treatment, production of IL-13 is seen, followed soon thereafter by the secretion of TGF-β_1 and the occurrence of fibrosis. Finally, we show that the latter requires IL-13 signaling through the IL-13Rα_2 and that the fibrosis can be treated separately from the inflammation by targeting IL-13 or TGF-β_1. Thus, the latter emerge as a common axis of cytokines leading to collagen deposition in inflamed organs.

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[2] Abbreviations used in this paper: CD, Crohn's disease; TNBS, 2,4,6-trinitrobenzene sulfonic acid; siRNA, small interfering RNA; HVJ-E, HVJ envelope; LPMC, lamina propria mononuclear cell; pSmad3, phosphorylated Smad3.

Materials and Methods

Mice

Female BALB/c mice (8–10 wk old) were obtained from The Jackson Laboratory and then maintained in the National Institute of Allergy and Infectious Diseases animal holding facilities. Female BALB/c CD1d-deficient mice were provided by Dr. J. Berzofsky (National Cancer Institute, National Institutes of Health, Bethesda, MD) and then housed at Biocon Animal Holding Facility (Rockville, MD). Animals were used according to the National Institutes of Health Laboratory Animal Care Guidelines.

Construction of pCI-sIL-13Rα_2-Fc

First, IL-13Rα_2 cDNA was inserted into pCI-Mammalian Expression Vector (Promega). In a second step, the cDNA encoding for the Fc part of a human IgG was inserted after the IL-13Rα_2 cDNA to connect both inserts resulting in a soluble form of the IL-13Rα_2.

IL-13Rα_2-specific small interfering RNA (siRNA)

IL-13Rα_2-specific siRNA and control (scrambled) siRNA for use in gene-silencing studies was obtained from Dharmacon. The mRNA-targeting sequence was as follows: 5′-GGAATCTAATTTACAAGGA-3′. The specificity and efficiency of this siRNA has been demonstrated previously (6). For high-efficiency in vivo delivery of siRNA, the latter was first encapsulated in HVJ envelope (HVJ-E) as described previously (14). The encapsulated siRNA was then administered by intrarectal instillation every other day starting on day 35 after initiation of chronic colitis with TNBS.

In vitro cytokine culture

Abs for IL-23p19, IL-17A, IL-18, and control IgG used in this experiment were purchased from R&D Systems. The Ab used for blocking IL-12p35 was purchased from eBioscience.

Induction of colitis

Chronic TNBS colitis was induced by weekly administration of increasing doses of TNBS (Sigma-Aldrich) (1.5–2.5 mg in 45% ethanol). Mice were lightly anesthetized with isoflurane and then administered TNBS/ethanol per rectum via a 3.5 F catheter equipped with a 1-ml syringe; the catheter was advanced into the rectum until the tip was 4 cm proximal to the anal verge, at which time the TNBS was administered in a total volume of 150 μl. To ensure distribution of TNBS within the entire colon and cecum, mice were held in a vertical position for 30 s after the intrarectal injection.

Intranasal administration of plasmid DNA

We administered pCI-sIL-13Rα_2-Fc encoding for soluble IL-13Rα_2-Fc fusion protein by intranasal rout. We used 100 μg of plasmid in 20 μl of PBS/mouse every other day, starting on day 35.

Cell isolation and culture

Isolated colonic lamina propria mononuclear cells (LPMC) were isolated from colonic tissues as previously described (8) and then cultured for 48 h. The cultured LPMC were stimulated with plate-bound anti-CD3-Ab (10 μg/ml) and soluble anti-CD28-Ab (1 μg/ml) (BD Pharmingen) to determine IFN-γ, IL-4, IL-13, and IL-17 protein secretion. To determine IL-12p70, TNF-α, and IL-18 protein secretion the cultured LPMC were stimulated with *Staphylococcus aureus* Cowan I (1/10,000 dilution of Pansorbin) (EMD Biosciences) and IFN-γ (1,000 U/ml) (R&D Systems). To determine IL-23 protein secretion the cultured LPMC were stimulated with peptidoglycan (100 μg/ml) (Sigma-Aldrich), and to determine TGF-β_1 protein secretion the LPMC were stimulated with IL-13 (20 μg/ml) plus TNF-α (20 μg/ml) (PeproTech).

Extraction of total colonic protein

Snap-frozen colonic samples were mechanically homogenized in liquid nitrogen, and total protein extracts were collected in buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 0.2 mM EGTA, supplemented with 1 mM DTT, 10 mg/ml leupeptin, and 1 mM phenylmethanesulphone. Extracts containing 100 mg of proteins were then analyzed for cytokine expression by cytokine-specific ELISA kits according to the manufacturer's instructions.

Assays of culture supernatants by ELISA

Cytokine concentrations of culture supernatants were measured by ELISA according to the manufacturer's instructions. ELISA kits were purchased for the following: IFN-γ, IL-4, IL-12p70, TNF-α, and IL-18 (BD Pharmingen); TGF-β_1 (BioSource International); IL-23 (eBioscience); and IL-17 (R&D Systems). TGF-β_1 was measured in medium containing TGF-β_1-depleted human serum.

Western blot analyses

Colonic LPMC were isolated and stimulated for 16 h with plate-bound anti-CD3 Abs. The cells were then lysed by radioimmunoprecipitation assay buffer and whole lysates obtained were subjected to SDS-PAGE, after which the separated proteins obtained were transferred to a nitrocellulose membrane and immunoblotted. IL-13Rα_2 was detected by incubation with a monoclonal rat anti-mouse IL-13Rα_2 (R&D Systems) followed by incubation with HRP-conjugated anti-rat IgG (Zymed Laboratories). Phosphorylated Smad3 (pSmad3) and total Smad3 were detected by incubation with a polyclonal rabbit-anti-phospho-Smad3 ($Ser^{433/435}$)-Ab (Cell Signaling Technology) and a polyclonal rabbit-anti-Smad2/3-Ab (Santa Cruz Biotechnology), respectively, followed by incubation with HRP-conjugated anti-rabbit IgG (Cell Signaling Technology). Smad7 was detected by incubation with a polyclonal goat-anti-phospho-Smad7 Ab (Santa Cruz Biotechnology) followed by incubation with HRP-conjugated anti-goat IgG (Zymed Laboratories). Membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to x-ray film.

RT-PCR

Aliquots of LPMC were stored in RNAlater solution (Ambion) and then subjected to RNA extraction using RNeasy tissue kit (Qiagen). A total of 1 μg of template RNA was reverse transcribed using the SuperScript III RT-PCR kit (Invitrogen Life Technologies). Primer sequences were as follows: IL-13Rα_1, 5′-GCAGCCTGGAGAAAAGTCGTCAAT-3′ and 5′-ACAGCCTCGGCAAGAACACCA-3′ and GAPDH, 5′-GGTGAAGGTCGGTGTGAACGGA-3′ and 5′-TGTTAGTGGGGTCTCGCTCCTG-3′. Annealing temperature and cycle number was as follows: IL-13Rα_1, 60°C and 27 cycles; GAPDH, 60°C and 25 cycles.

Collagen assay

Colons of TNBS-treated mice were harvested on day 49 of study and homogenized in 0.5 M acetic acid containing 1 mg of pepsin (at a concentration of 10 mg of tissue/10 ml of acetic acid solution). The resulting mixture was then incubated and stirred for 24 h at 4°C. Total soluble collagen content of the mixture was then determined with a Sircol Collagen Assay Kit (Biocolor) (15). Acid soluble type I collagen supplied with the kit was used to generate a standard curve.

Assay of activated AP-1 components

Nuclear extracts from colonic LPMC were obtained using the Transfactor Extract Kit (Active Motif). The extracts were then tested for DNA binding activity using the c-jun TransAM Kit (Active Motif) according to the manufacturer's instructions. In brief, nuclear extract (15–30 μg) was applied to each well coated with AP-1 consensus oligonucleotides, and then wells were incubated with specific Abs for c-jun followed by HRP-labeled secondary Abs. Color development obtained by adding tetramethylbenzidine substrate was stopped by adding H_2SO_4, at which point absorbance was measured at 450 nm wavelength (6, 10, 16).

Histological examination of colonic tissue and scoring of inflammation

Colons were fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded colon sections were cut and then stained by the Masson's trichrome method. For calculation of inflammation indices or for assessment of fibrosis in the treated and control group of mice, the sections were read blindly and evaluated according to a previously described scoring system (17).

Statistical analyses

Student's t test computed by GraphPad Prism 4 was used to evaluate the significance of the differences. A value of $p < 0.05$ was considered statistically significant.

Results

Clinical features of chronic TNBS-induced colitis in BALB/c mice

To establish a chronic form of TNBS colitis suitable for the study of inflammation-induced colonic fibrosis, we administered repeated (weekly) intrarectal doses of TNBS in ethanol to BALB/c

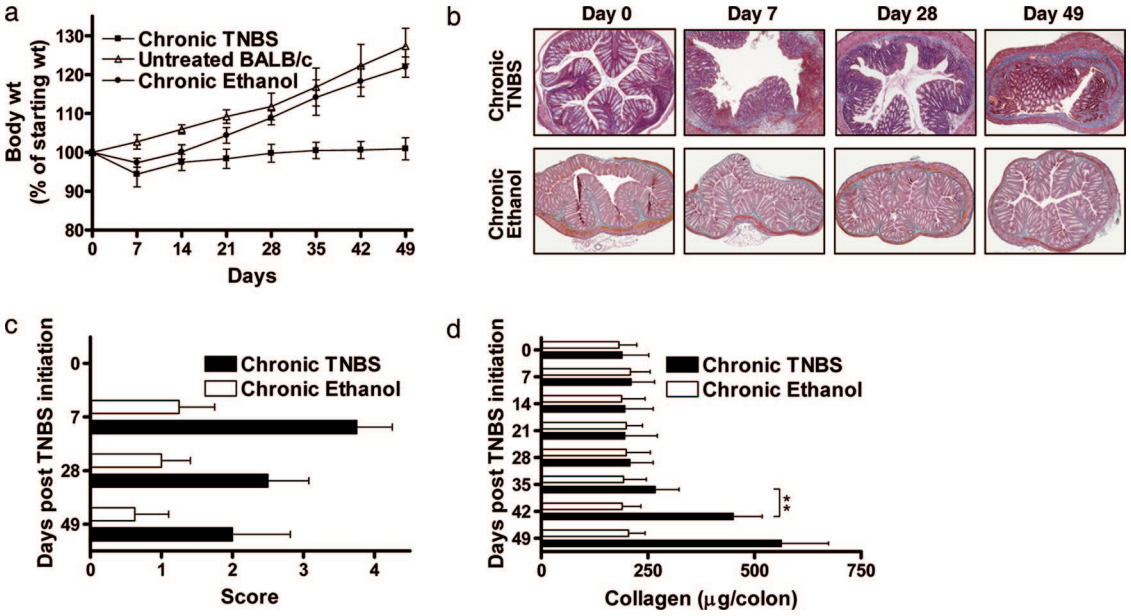

FIGURE 1. Clinical features of chronic TNBS colitis in BALB/c mice. *a*, Body weight as a percentage of starting weight. Data shown are mean values ± SD derived from at least five mice per group. *b*, Masson's trichrome staining of representative colon cross-sections during chronic TNBS colitis and chronic ethanol administration (magnification, ×5). *c*, Histological scores shown are mean values ± SD from at least five mice per group. *d*, Collagen content of the colon. Collagen content was determined during chronic TNBS colitis and chronic ethanol administration by a Sircol assay (see *Materials and Methods*). Data shown are mean values ± SD derived from at least five mice per group; **, $p < 0.01$.

mice following a modified protocol recently reported by Lawrance et al. (13). As shown in Fig. 1*a*, after the administration of the first dose of TNBS the mice lost ~5–10% of their body weight and exhibited ruffled coats, hunched posture, and restricted movement; in addition, ~10–15% of animals died during this period. However, during the course of the next 4–5 wk, the surviving mice

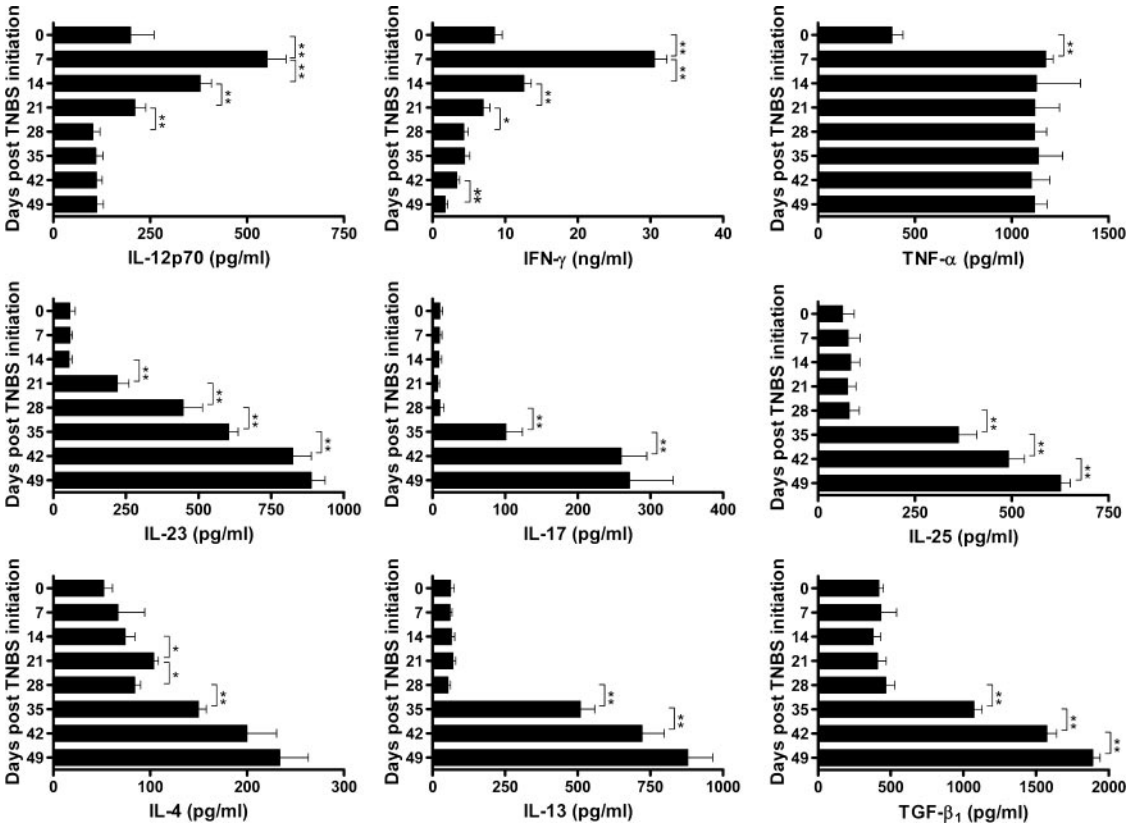

FIGURE 2. Cytokine production of colonic LPMC at weekly time points during chronic TNBS colitis in BALB/c mice. LPMC were extracted from the lamina propria and cultured for 48 h in the presence of T cell or APC stimulants (see *Materials and Methods*). Cytokine concentrations in the culture supernatants were determined by cytokine-specific ELISA. Data shown are mean values ± SD from individual cultures of cells derived from mice in two separate experiments, each containing at least five mice per group; *, $p < 0.05$ and **, $p < 0.01$.

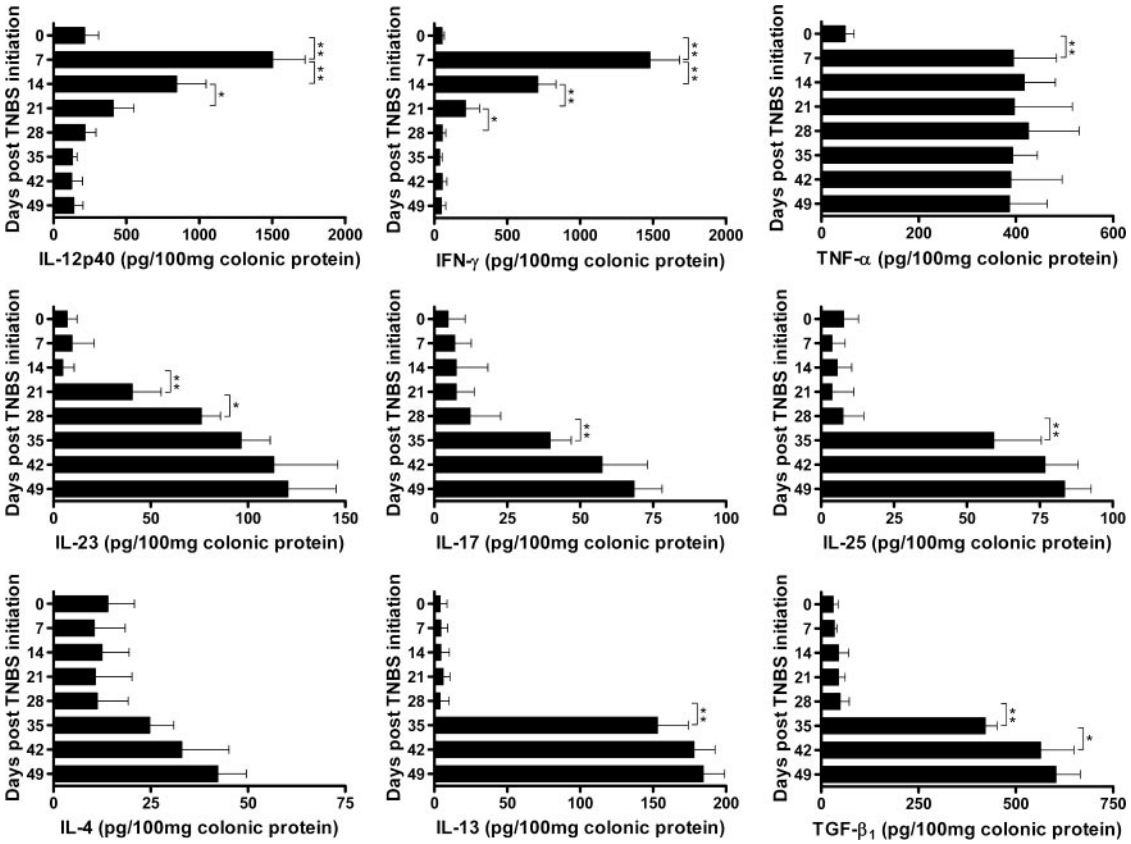

FIGURE 3. In situ cytokine production in the colonic mucosa of mice with chronic TNBS colitis measured at weekly time points. Total colonic protein was extracted from mice in the various groups and subjected to cytokine-specific ELISAs. Data shown are representative of two independent experiments each containing at least five mice per group. Data shown are mean values ± SD derived from at least five mice per group; *, $p < 0.05$ and **, $p < 0.01$.

gradually regained lost weight and recovered from other obvious signs of chronic illness despite continued TNBS administration; thus, during this period, only their failure to match the natural weight gain of littermate controls not administered TNBS, remained as an outward manifestation of continued colonic inflammation. Chronic administration of intrarectal ethanol to mice alone led to a weight loss of up to 5% in the early phase of the disease; however, after this period, the mice gained weight almost to the extent of their littermate controls.

As shown in Fig. 1, *b* and *c*, the above "clinical" response to chronic TNBS administration correlated with serial histologic examination and histologic scoring of colonic tissue: during the first 1–2 wk of disease, the lamina propria of the colon was characterized by a massive infiltration of inflammatory cells, and evidence of epithelial cell disruption was similar to that seen in conventional TNBS colitis. However, the level of inflammatory cell infiltration, as well as the areas with epithelial cell destruction decreased during the third through fifth week. In addition, at this point edema of the colonic wall became evident. Finally, during the sixth and seventh week of the disease, the lamina propria showed pockets of inflammation alternating with areas of minimal or moderate inflammation as well as persistent edematous swelling of the colonic wall. Moreover, during this phase the colon exhibited obvious collagen deposition in the subepithelial areas and in the serosal areas, especially in the distal colon. In contrast, mice administered intrarectal ethanol alone exhibited only minimal inflammatory changes only in the first 2 wk after initial TNBS instillation and did not develop colonic fibrosis.

To quantitate the amount of the intestinal fibrosis present in the lamina propria over the course of the disease, we determined the amount of collagen present in the tissue with a Sircol assay (see *Materials and Methods*). As shown in Fig. 1*d*, we found no increase in tissue collagen content (compared with controls) during the first 4 wk of colitis. However, at the end of the fifth week a slight (statistically nonsignificant) increase was noted, and in the sixth and seventh week large statistically significant increases were observed. Tissue from mice administered ethanol alone did not exhibit an increase in colonic collagen content.

Changes in cytokine expression during the course of disease

In further studies, we determined the cytokines profile of LPMC during the course of the chronic TNBS colitis. Accordingly, we isolated colonic LPMC from mice at weekly intervals throughout the course of study (7 wk) (see *Materials and Methods*), stimulated the isolated cells for 48 h, and measured the specific cytokine concentrations in the culture supernatants by ELISA (see *Materials and Methods*).

As shown in Fig. 2 and as anticipated from previous experience with acute TNBS colitis, we noted that the production of Th1 cytokines, IL-12p70, and IFN-γ by extracted cells were greatly elevated on day 7 after initiation of TNBS administration. However, surprisingly, the production of these cytokines gradually decreased over the next 2 wk, returning to baseline levels by day 21 and then gradually declining to levels increasingly below that observed at baseline. In contrast, production of TNF-α, a cytokine appearing in both Th1- and Th2-mediated inflammation, was elevated on day 7 and remained elevated throughout the course of the study.

Interestingly, the decline in the production of IL-12p70 and IFN-γ coincided with an increased production of an alternative set

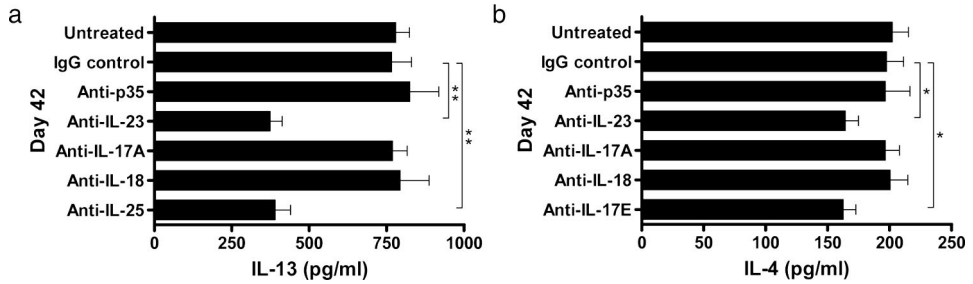

FIGURE 4. IL-13 (*a*) and IL-4 (*b*) production by isolated colonic LPMC stimulated in vitro in the presence of anti-cytokine Abs. Cells were extracted from the lamina propria on day 42 of chronic TNBS colitis and cultured for 48 h in the presence of plate-bound anti-CD3 and soluble anti-CD28 as well as specific blocking Abs or control IgG. Cytokine concentrations in the supernatants were determined by ELISA. *, $p < 0.05$ and **, $p < 0.01$.

of proinflammatory cytokines, namely IL-23 and IL-17. Increased IL-23 production above baseline was noted first, on day 21, and thereafter increased further until it reached a plateau on day 42. Increased IL-17 production was noted somewhat later, on day 35, and thereafter also increased further until it reached a plateau on day 42. These sequential changes may reflect the fact that IL-23 may be necessary for sustained IL-17 production (18).

Somewhat surprisingly, increased production of IL-23 and IL-17 coincided with increased production of cytokines usually considered Th2 cytokines. Thus, beginning on day 21 there was a gradual increase in IL-4 production that, on day 35, was accompanied by an abrupt increase in IL-13 production. Production of each of these cytokines increased each week after their first appearance but appeared to be leveling off in the last week of the study on day 42. Whereas IL-4 production never achieved levels equivalent to those seen in a polarized Th2 response, IL-13 production did achieve such levels; on this basis, one could say that the IL-13 component of the response was the more dominant component of the response (8, 10).

Previous studies have shown that IL-25, a member of the IL-17 family of proteins (and designated IL-17E), is associated with expression of Th2 cytokines, at least when it is overexpressed in transgenic mice (19). We therefore measured the level of IL-25 in the present inflammation model. As shown in Fig. 2, "background" IL-25 levels could be detected in naive mice on day 0 and remained background until day 28. However, on day 35 a massive increase in IL-25 secretion appeared and thereafter IL-25 secretion further increased, reaching its highest level on day 49.

As shown in Fig. 3, the above pattern of cytokine expression obtained from studies of isolated colonic LPMC stimulated in vitro

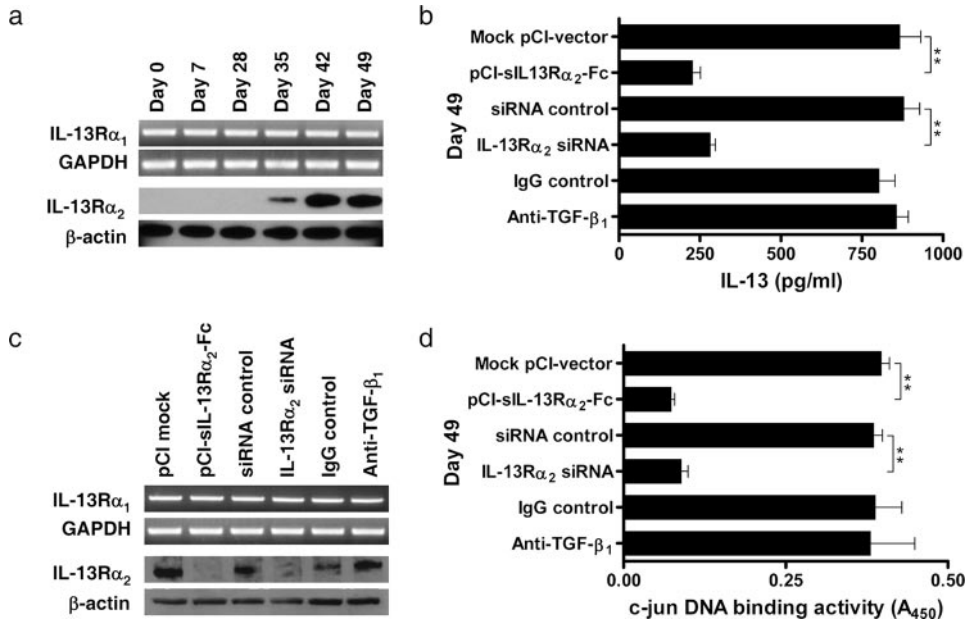

FIGURE 5. IL-13Rα_1 and IL-13Rα_2 expression and function in BALB/c mice during the course of chronic TNBS colitis. *a*, IL-13Rα_1 expression is constitutive whereas IL-13Rα_2 expression is induced on day 35 during chronic TNBS colitis. IL-13Rα_1 mRNA expression was determined by RT-PCR of RNA extracted from colonic LPMC, and IL-13Rα_2 protein expression was determined by Western blot analysis analyzed of colonic LPMC lysates. *b*, IL-13 production after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1-Ab. Cells were extracted from the lamina propria and cultured for 48 h in the presence of plate-bound anti-CD3 and soluble anti-CD28. Cytokine concentration was determined in culture supernatants by ELISA. Data shown are mean values ± SD for the pooled data derived from two separate experiments. Groups in each experiment contained at least five mice; **, $p < 0.01$. *c*, IL-13Rα_2 expression (but not IL-13Rα_1) is reduced in chronic TNBS after treatment with pCI-sIL-13Rα_2-Fc and IL-13Rα_2-specific siRNA. IL-13Rα_1 and IL-13Rα_2 expression were determined as described in *a*. *d*, DNA-binding activity of c-jun in nuclear extracts of cells derived from isolated lamina propria is reduced after administration of pCI-sIL-13Rα_2-Fc and IL-13Rα_2-specific siRNA but not anti-TGF-β_1-Ab. DNA-binding activity was determined in nuclear extracts from colonic lamina propria cells by the TransAM assay (see *Materials and Methods*). Data shown are mean values ± SD for the pooled data derived from three separate experiments. Groups in each experiment contained at least five mice; **, $p < 0.01$.

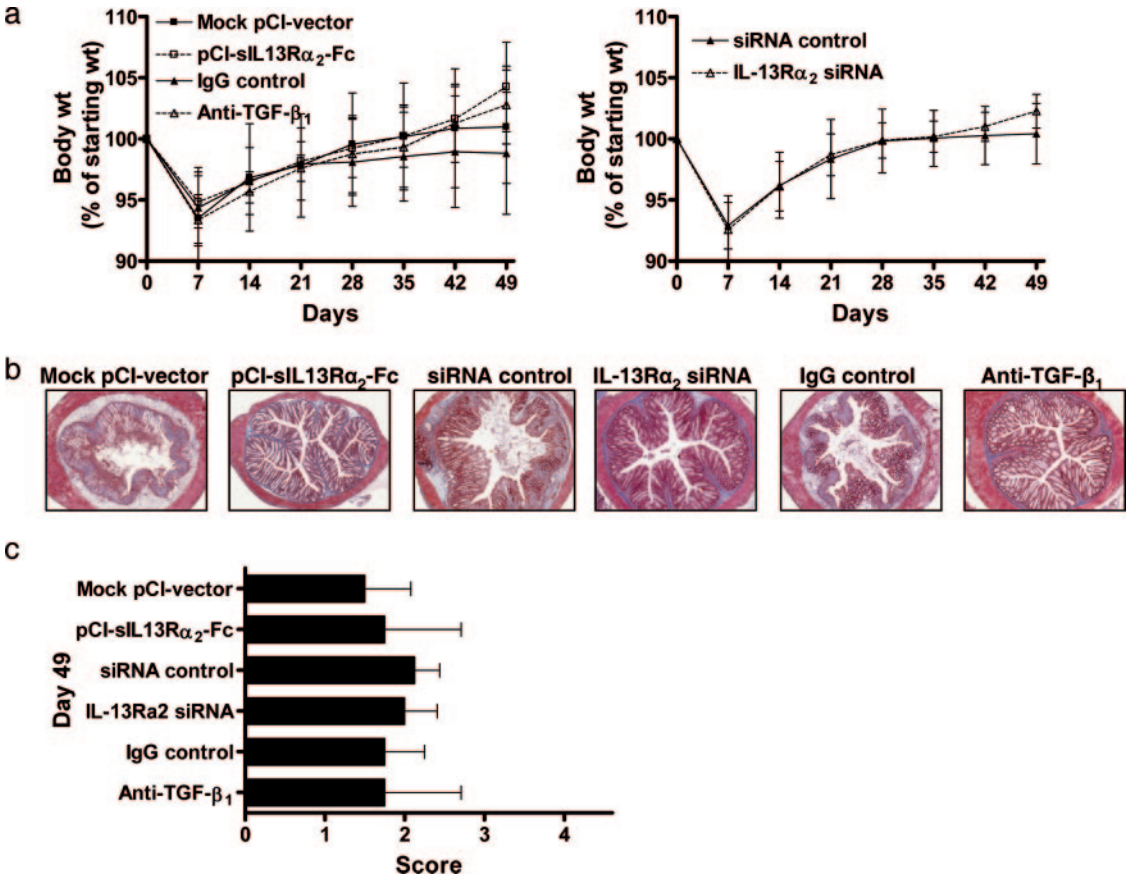

FIGURE 6. Body weight, collagen deposition, and histological scores of mice on day 49 of chronic TNBS colitis after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1 beginning on day 35 of chronic TNBS colitis. *a*, Body weight as a percentage of starting weight after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1-Ab (see text). Data shown are mean values ± SD derived from at least five mice per group. *b*, Masson's trichrome staining of representative colon cross-sections during chronic TNBS colitis after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1-Ab (magnification, ×5). *c*, Histological scores after administration of pCI-sIL-13Rα_2-Fc or anti-TGF-β_1-Ab. Scores shown are mean values ± SD from at least five mice per group.

was corroborated by studies of in situ cytokine levels in samples of total colonic protein extracts measured by ELISA.

Finally, as mentioned above, IL-13 has been shown to induce TGF-β_1 both in vitro and in vivo. Thus, it was not surprising that levels of TGF-β_1 increased in parallel with IL-13 levels.

Origin of the IL-13 response in chronic TNBS colitis

One of the surprising aspects of the above cytokine cascade in chronic TNBS colitis was the finding that an initial Th1 response culminated in the robust production of IL-13, normally a Th2 cytokine. To better understand this progression, we first performed in vitro studies to determine whether IL-13 production was induced by various cytokines that preceded it in order of appearance such as IL-12, IL-23, IL-17, IL-18, or IL-25/17E. Accordingly, we set up cultures of colonic LPMC obtained from mice on day 42 after the initiation of the study and determined the effect of Abs to these cytokines on the production of IL-13 in cells stimulated by anti-CD3/anti-CD28. As shown in Fig. 4*a*, addition of anti-IL-23 or anti-IL-25 partially inhibited IL-13 production, whereas anti-p35, anti-IL-17, or anti-IL-18 had no effect. In addition, as also shown in Fig. 4*b*, these Abs had minor (albeit statistically significant) effects on IL-4 production. Recognizing that many of the LPMC might have already been committed to IL-13 production, this partial inhibition suggests that the IL-13 response has its origin in a preceding IL-23/IL-25 response, but further in vivo studies would be necessary to confirm this supposition. If this is indeed the case, the IL-13 response may not be part of a Th2 response because in prior studies, it has been shown that IL-25-driven IL-13 responses originate from a poorly defined cell that is probably not a T cell (see further discussion below) (20).

In a second series of studies along these lines, we determined whether NKT cells were the source of the IL-4/IL-13. This question was prompted by previous studies of oxazolone colitis in which it was shown that a Th2 response mediated initially by IL-4 and later by IL-13 production was responsible for the inflammation; furthermore, in this case NKT cells rather than conventional T cells were the source of the IL-13. To determine whether NKT cells were producing IL-13 in chronic TNBS colitis, we determined cytokine production in chronic TNBS colitis established in BALB/c CD1-deficient mice, with the knowledge that such mice do not express NKT cells because the latter require Ag presented by the atypical MHC class I molecule, CD1, for their expansion (21–24). Thus, LPMC obtained from BALB/c mice lacking CD1 on day 49 of chronic TNBS colitis and stimulated in vitro with anti-CD3/anti-CD28 produced substantial amounts of IL-13 and collagen deposition, albeit ~20% less than control BALB/c mice with chronic TNBS colitis (data not shown). This mild reduction in IL-13 production in CD1-deficient mice is probably attributable to the fact that BALB/c CD1-deficient mice do not mount as strong a Th1 response as normal mice because NKT cells are probably necessary for optimal Th1 priming (22, 23).

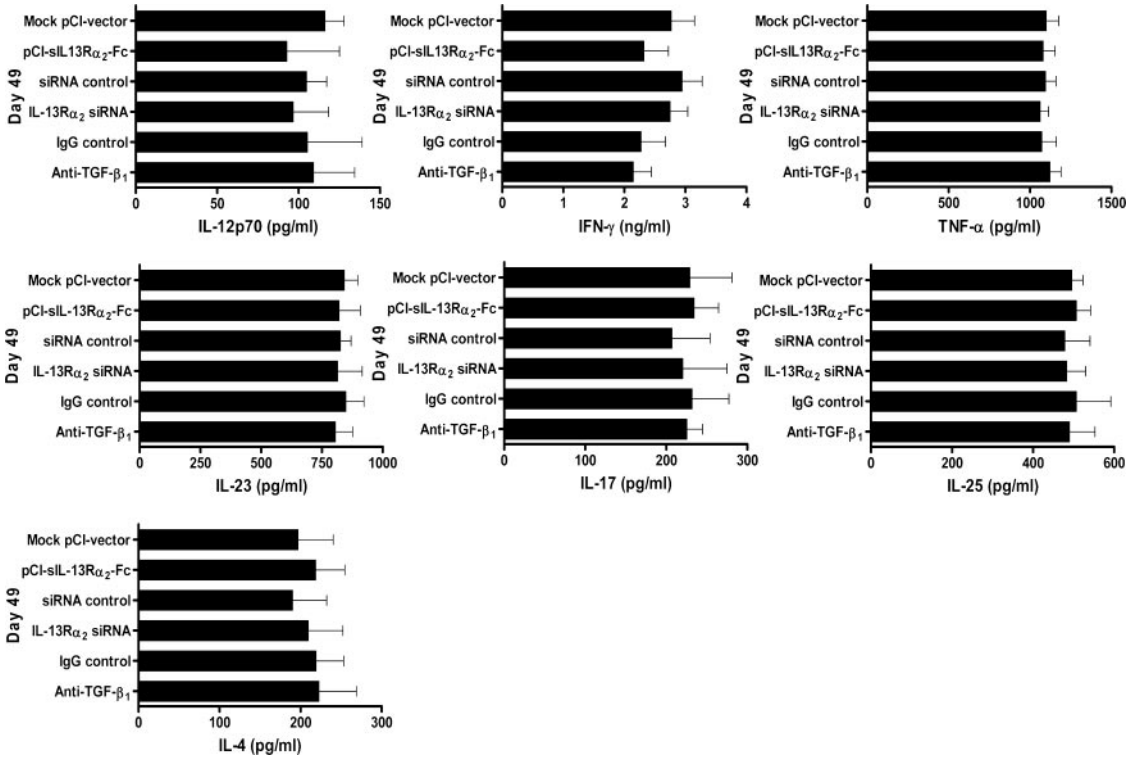

FIGURE 7. LPMC cytokine production of mice on day 49 of chronic TNBS colitis after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1 beginning on day 35 of chronic TNBS colitis. Cells were extracted from the lamina propria and cultured for 48 h in the presence of plate-bound anti-CD3 and soluble anti-CD28. Cytokine concentration was determined in culture supernatants by ELISA. Data shown are mean values ± SD from individual cultures of cells derived from mice in two separate experiments, each containing at least five mice per group. Note that the scales used in these panels are frequently different from those used in Fig. 2, due to the lower cytokine production at this time point.

IL-13 induces expression of, and signaling through, IL-13Rα_2 in the late phase of chronic TNBS colitis

In previous studies, we showed that IL-13 induces TGF-β_1 production by signaling through cell surface IL-13Rα_2, an IL-13R formerly thought to function only as a soluble decoy receptor (6, 25–27). We also showed the IL-13Rα_2 transduces activation of an AP-1 variant consisting of c-jun and Fra-2. Because IL-13 is produced in the later phase of chronic TNBS colitis, we reasoned that this pathway might be a key factor in the development of fibrosis occurring during this phase. To explore this possibility, we first determined the expression of both IL-13Rα_1 and IL-13Rα_2 in extracts of colonic LPMC during the course of chronic TNBS colitis. IL-13Rα_1 expression was determined by RT-PCR (because no Abs suitable for Western blot are available), and IL-13Rα_2 expression was determined by Western blot analysis. As shown in Fig. 5*a*, IL-13Rα_1 was constitutively expressed because it was detectable at every measured time point and revealed no changes during the course of the inflammation. In contrast, IL-13Rα_2 was an induced receptor because it was not evident in naive mice or in the first few weeks of chronic TNBS colitis, but could be detected initially at a low concentration on day 35 and later at a high concentration on days 42 and 49 after initiation of colitis.

The above time course of IL-13Rα_2 expression correlated with that of IL-13 and was thus consistent with the possibility that the latter was an inducing cytokine. To address this question more directly, we next blocked IL-13 activity by administration of a plasmid (pCI-sIL-13Rα_2-Fc) encoding a soluble IL-13Rα_2-Fc fusion protein (pCI-sIL-13Rα_2-Fc) or a control plasmid (pCI empty vector). The latter was delivered by the intranasal route every other day starting on day 35 after initiation of chronic colitis (28). We verified that intranasal administration of the sIL-13Rα_2-Fc plasmid results in expression of sIL-13Rα_2-Fc in colonic tissue by demonstrating that human Ig-Fc (a component of the plasmid product) is expressed in colonic tissue extracts following administration of the plasmid (data not shown). As shown in Fig. 5*b*, the administration of the pCI-sIL-13Rα_2-Fc plasmid by this route, but not the control plasmid, led to a reduction in LPMC production of IL-13. This effect is consistent with a previous study by Chiaramonte et al. (25) who showed that mice with IL-13Rα_2 receptor deficiency have reduced IL-13 production and is possibly based on the fact that autocrine IL-13 signaling via this receptor is necessary for optimal IL-13 production. In addition, as shown in Fig. 5, *c* and *d*, administration of pCI-sIL-13Rα_2-Fc led to a virtually complete loss of IL-13Rα_2 expression in extracts of colonic tissue obtained at the conclusion of the study (day 49) as well as loss of expression of c-Jun in the same extracts, a factor shown to be induced by IL-13Rα_2 signaling. In contrast, neutralization of TGF-β_1 by administration of anti-TGF-β_1 had no effect on receptor expression or signaling; this result was expected because there is no evidence to suggest that TGF-β_1 induces up-regulation of IL-13Rα_2. Taken together, these results strongly suggest that IL-13 production occurring in the late phase of chronic TNBS colitis is indeed responsible for the expression of IL-13Rα_2.

Blocking of the IL-13 signaling and resultant TGF-β_1 responses prevents the development of intestinal fibrosis in chronic TNBS colitis

In previous studies, we have shown that that intrarectal administration of NF-κB decoy oligonucleotides on days 35 and 42 abrogates the inflammation and the accompanying cytokine response that are characteristic of the chronic inflammation at this stage of the inflammation, including the high levels of IL-23, IL-17, and

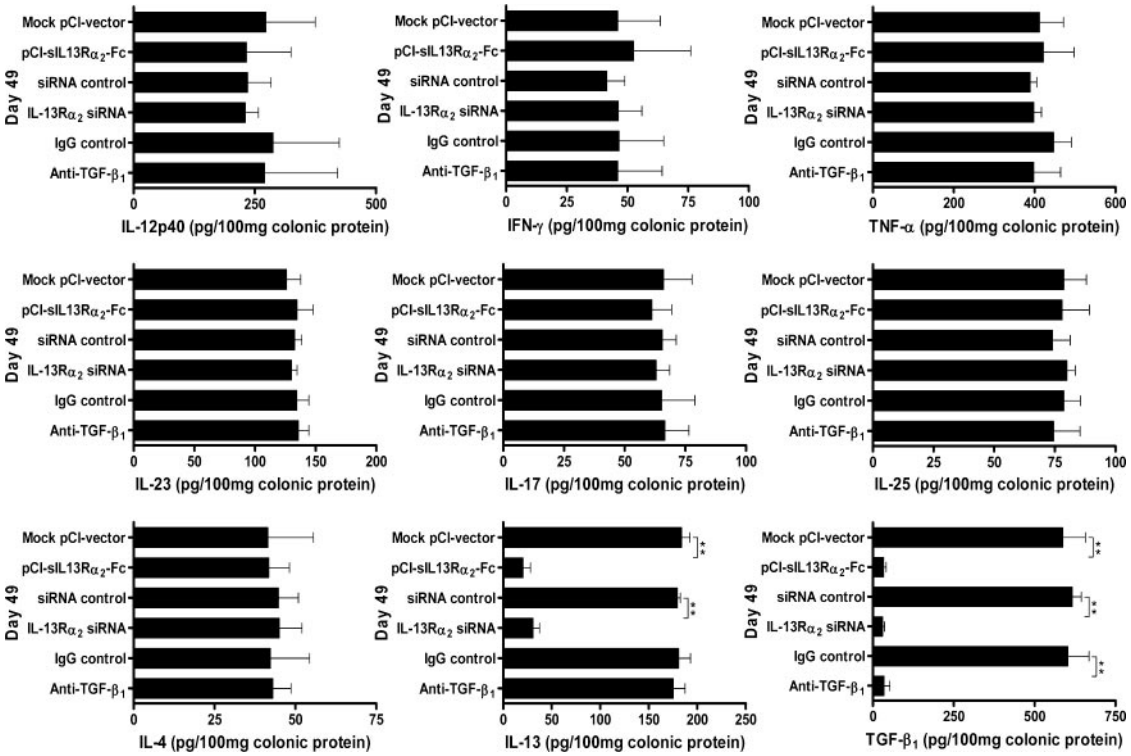

FIGURE 8. In situ cytokine production in the colonic mucosa of mice with chronic TNBS colitis measured after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1-Ab. Total colonic protein was extracted from mice in the various groups and subjected to cytokine-specific ELISAs. Data shown are mean values ± SD derived from at least five mice per group; **, $p < 0.01$.

IL-13 (10). In addition, such treatment prevented the production of TGF-β_1 and the development of intestinal tissue fibrosis. However, whereas these data established that the IL-13 and TGF-β_1 responses as well as the collagen deposition were an outgrowth of the inflammation, they did not address the question of whether or not IL-13 and/or TGF-β_1 were the cause of such deposition.

To address this point in the present study, we determined the effect of in vivo inhibition of either IL-13 signaling or TGF-β_1 activity on the generation of tissue fibrosis. In these studies we inhibited IL-13 signaling in two ways: 1) by intranasal administration of the plasmid (described above) encoding a soluble IL-13Rα_2-Fc fusion protein (pCI-sIL-13Rα_2-Fc) or a control plasmid (pCl empty vector) to block the receptor with the use of a decoy; and 2) by intrarectal administration of IL-13Rα_2-specific siRNA encapsulated in HVJ-E or a control siRNA to down-regulate the receptor by gene silencing. Both the sIL-13Rα_2-Fc plasmid and the siRNA were administered every other day starting on day 35 after the initiation of the chronic inflammation. In contrast, for study of the effects of inhibition of TGF-β_1, we administered anti-TGF-β-Ab or isotype control Ig by an i.p. route over the same time period. The effects of these treatments on IL-13 and TGF-β_1 levels were subsequently determined at the termination of the study on day 49. For this purpose, LPMC extracted from the colons of sacrificed mice were cultured for 48 h with anti-CD3/anti-CD28, and their secretion of IL-13 and TGF-β_1 into the culture supernatant was measured by ELISA.

As shown in Fig. 6, neutralization of IL-13 signaling by fusion protein or gene silencing of IL-13Rα_2 by siRNA as well as neutralization of TGF-β_1 by Ab was accompanied by only a small, nonsignificant increase in body weight and no measurable change in colitis score. In addition, these treatments were not accompanied by changes in inflammatory cytokine secretion. Thus, as shown in Fig. 7, LPMC extracted from the colons of sacrificed mice on day 49 and evaluated for secretion of cytokines secreted equal amounts of IL-12p70, IFN-γ, TNF-α, IL-23, IL-17, IL-25, and IL-4, whether or not they were subjected to neutralization of IL-13 signaling or TGF-β_1. As shown in Fig. 8, the above pattern of cytokine expression obtained from studies of isolated colonic LPMC stimulated in vitro was corroborated by studies of in situ cytokine levels in samples of total colonic protein extracts measured by ELISA. In contrast, a very different picture was obtained with respect to TGF-β_1 secretion. In this case, as shown in Fig. 9*a*, the cells extracted from the colons of mice subjected to neutralization of IL-13 signaling by fusion protein or IL-13Rα_2 down-regulation by siRNA exhibited greatly reduced production of TGF-β_1. In addition, as shown in Fig. 9*b*, those mice subjected to neutralization of IL-13 signaling as well as those subjected to TGF-β_1 neutralization displayed basal collagen levels seen in naive mice, whereas those mice not subjected to such neutralization displayed undiminished collagen levels as compared with other mice with chronic TNBS colitis. Overall, these data show quite clearly that neutralization of IL-13 signaling via IL-13Rα_2 does not alter the course of the inflammation of chronic TNBS colitis but does block the development of fibrosis; in addition, they show that such signaling results in fibrosis via its effect on the induction of TGF-β_1.

Smad3 phosphorylation and Smad7 expression in chronic TNBS colitis with and without neutralization of IL-13 and TGF-β_1

Previous studies have shown that TGF-β_1 induces collagen production through a Smad2/Smad3-dependent signaling pathway (29–33). To determine whether this might also occur in chronic TNBS colitis, we obtained whole cell lysates from LPMC that had been cultured for 16 h with anti-CD3/anti-CD28 and then subjected the lysates to Western blot analyses of activated Smad proteins. As shown in Fig. 10*a*, whereas unphosphorylated Smad2/3 was detected in Western blots of extracts obtained from cells at

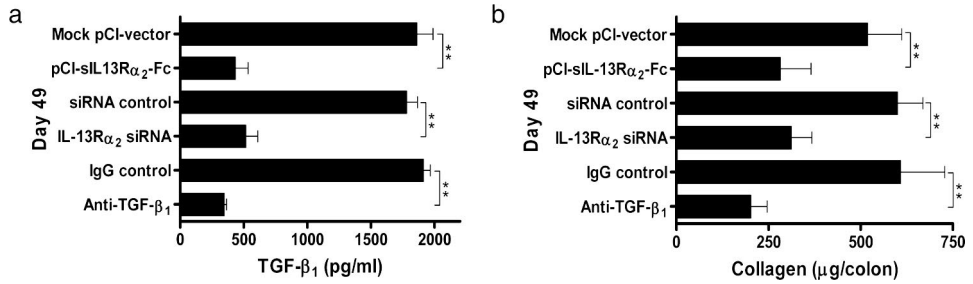

FIGURE 9. TGF-β_1 secretion by colonic lamina propria cells on day 49 of chronic TNBS colitis in BALB/c mice. *a*, TGF-β_1 secretion after treatment with pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1-Ab. Cells were extracted from the lamina propria and cultured for 48 h in the presence of stimulants (see *Materials and Methods*). Cytokine concentration was determined in supernatants by ELISA. Data shown are mean values ± SD from two separate experiments. Groups in each experiment contained at least five mice; **, $p < 0.01$. *b*, Collagen content of the colon after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1-Ab. Collagen content was determined during chronic TNBS colitis by a Sircol assay. Data shown are mean values ± SD derived from at least five mice per group; **, $p < 0.01$.

any of the investigated time points, pSmad3 was not seen until days 35 and at an increased level on days 42 and 49, the time points at which colonic LPMC manifested greatly increased TGF-β_1 production. We can thus conclude that TGF-β_1 production in chronic TNBS colitis was probably accompanied by signaling of cells via Smad activation.

In further studies we determined the expression of Smad7, a Smad molecule known to down-regulate TGF-β_1 signaling via inhibition of Smad2/3 activation (34–36). As also shown in Fig. 10*b*, Smad7 was produced during the early stages of chronic TNBS colitis (on days 7 and 35) but was not produced during the later stage when TGF-β_1 production was being induced and fibrosis was occurring. Although it is not clear why Smad7 was not evident during the later stage of chronic TNBS colitis, it is clear that Smad7 was not acting as an inhibiting factor in TGF-β_1-mediated fibrosis.

As shown above, IL-13Rα_2 expression and subsequent TGF-β_1 induction is down-regulated by the inhibition of IL-13 with administration of pCI-sIL-13Rα_2-Fc as well as with IL-13Rα_2-specific siRNA, and, in addition, TGF-β_1 signaling is down-regulated by the administration of anti-TGF-β_1. We therefore anticipated that the induction of Smad3 phosphorylation is also inhibited by administration of these reagents. As shown in the Western blot shown in Fig. 10*c*, this result proved true in that specific inhibition of IL-13 or TGF-β_1 led to diminished Smad3 phosphorylation, but not to an effect on total Smad2 or Smad3. As shown in Fig. 10*d*, Smad7 could not be detected in the final phase of the chronic inflammation, regardless of whether or not IL-13 and TGF-β_1 had been neutralized.

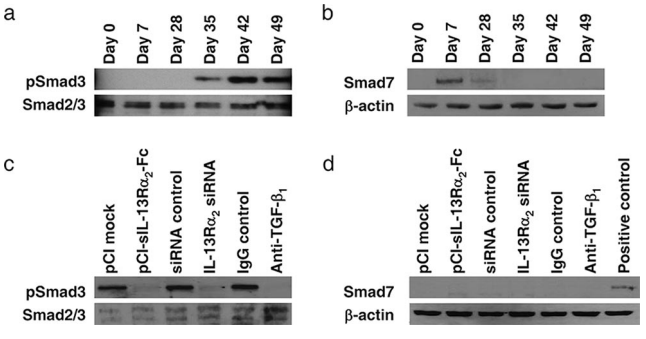

FIGURE 10. Smad protein expression during the course of chronic TNBS colitis in BALB/c mice. *a*, pSmad3 is induced in the late phase (Day 35) of chronic TNBS colitis. pSmad3 in colonic LPMC lysates were analyzed by Western blot. *b*, Smad7 is expressed on day 7 and day 28 of chronic TNBS colitis. Smad7 level in colonic LPMC lysates was analyzed by Western blot. *c*, Expression of pSmad3 on day 49 of chronic TNBS colitis is inhibited after administration of pCI-sIL-13Rα_2-Fc, IL-13Rα_2-specific siRNA, or anti-TGF-β_1-Ab. pSmad3 levels in colonic LPMC lysates were analyzed by Western blot. *d*, Smad7 is not expressed on day 49 of chronic TNBS colitis. Smad7 in colonic LPMC lysates was analyzed by Western blot.

Discussion

Several years ago, Lawrance et al. (13) reported that one can establish a chronic hapten-induced colitis in BALB/c mice by the repeated intrarectal administration of TNBS and that such colitis was ultimately accompanied by the occurrence of gastrointestinal fibrosis. In this study, we defined the cytokine milieu that characterizes various stages of this colitis, with the view of identifying the cytokines and cytokine signaling that is responsible for the fibrosis.

The course of chronic TNBS colitis can be divided into three distinct phases. The first phase, lasting from day 0 until approximately day 14, is characterized by an acute inflammation leading to extensive tissue damage and inflammatory cell infiltration; this phase is associated with and probably driven by high levels of IL-12p70 and IFN-γ that peak on day 7. A mortality rate of ~10–15% accompanies this early stage of the disease. The next phase lasting from about day 14 through day 35 is marked by continued but nonprogressive inflammation that allows the mice to maintain but not increase body weight. During this phase, IL-12p70 and IFN-γ levels decline and are replaced by gradually increasing IL-23 levels as well as increasing levels of IL-23-inducible cytokines such as IL-17 and IL-25/IL-17E. The third and last phase of the chronic intestinal inflammation is a somewhat overlapping phase that starts on day 28 and continues through day 49. This phase is characterized by slowly increasing deposition of collagen accompanied by secretion of cytokines usually considered to be Th2 cytokines, including low levels of IL-4 and high levels of IL-13; in addition, TGF-β_1 secretion, the likely source of increased collagen deposition, is seen during this period. This switch in cytokine profile from one dominated by IL-12p70 and IFN-γ to a cytokine profile dominated by IL-23 and IL-17, characterized by cytokines usually considered to be part of the Th2 response, is reminiscent of the switching cytokine profiles seen previously in the colitis of the IL-10 knockout mouse and the SAMP1/Yit mouse (37, 38).

The complex cytokine profile seen in chronic TNBS colitis relates to recent studies showing that IL-17 and IL-23 are important components of inflammatory reactions in several murine models of inflammation, including experimental allergic encephalomyelitis,

collagen-induced arthritis (39, 40), and, most importantly to the present discussion, in cell transfer and pathogen-induced colitis (41, 42). IL-23 is a member of the IL-12 cytokine family in that it shares the p40 chain of IL-12p70, but it is paired with a p19 chain rather than a p35 chain (as in IL-12p70) (43–45). Initially, it was thought that IL-23 induces Th17 cells producing IL-17 in parallel with the ability of IL-12p70 to induce Th1 cells producing IFN-γ. However, it is now known that IL-17-producing cells are actually induced by TGF-β_1 and IL-6 and IL-23 influences the IL-17 response secondarily, probably by acting on newly formed IL-17-producing cells to maintain their growth and/or survival (18). Taking advantage of the structural difference between IL-12p70 and IL-23, it has been possible to show that mice deficient in the p19 chain, and thus unable to produce IL-23 and maintain IL-17 responses, develop little or no colitis in the cell transfer colitis model, whereas mice deficient in the p35 chain, and unable to produce IL-12p70, continue to have severe colitis in this model (46). It should be noted, however, that there is evidence that this IL-12/IL-23 dichotomy is not complete because some IFN-γ is produced in mice lacking p35 and that IFN-γ contributes to inflammation in a *H. hepaticus*-induced colitis model (41, 42). In addition, in the hapten-induced model of colitis, IL-12p70 appears to be more important than IL-23 and, in fact, is more severe in the absence of the latter cytokine (47). Thus, it is still somewhat unclear whether IL-12p70 or IL-23 is more important or indeed whether both are important in human disease (48).

In the present study of chronic TNBS colitis, we again see a somewhat different picture than that seen in cell transfer colitis in that the IL-12 and IL-23 responses occur sequentially rather than simultaneously and the IL-12p70 response is clearly capable of inducing severe inflammation in the absence of the IL-23 response; in fact, in this case, the IL-23 response ushers in a relatively stable and relatively moderate level of inflammation. This sequential cytokine production pattern seen in chronic TNBS colitis suggests that Th1 and Th17 T cells predominate at different stages of mucosal inflammation that are ordinarily represented by (acute) TNBS colitis and cell transfer colitis, respectively.

Another distinct feature of the cytokine milieu in chronic TNBS colitis is that IL-23 production (and/or IL-25 production) appeared to induce or at least facilitate cytokine production usually seen during Th2 responses, i.e., IL-4 and IL-13 production. This is suggested by the timing of the responses as well as in vitro data, showing that the production of IL-13 can be partially inhibited by Abs to IL-23 and IL-25 but not by Abs to IL-12p70 (anti-p35). However, in vivo studies with blocking Abs will be necessary to address the question of apparent IL-23/IL-25 linkage to Th2 cytokines more definitively. A further point is that in previous studies of IL-25 responses, it has been shown that whereas this cytokine induces the production of IL-13, the cellular source of the IL-13 was not a T cell because it occurs to an equal extent in RAG2-deficient mice (20). This result suggests that the IL-13 induced in the chronic TNBS colitis model studied here is not in fact part of a Th2 response at all, but rather a response arising from an APC, such as a dendritic cell. This is supported by the finding that stimulation of dendritic cells with TLR2 ligands leads to IL-13 production (49).

Turning now to the main focus of the present study, namely the pathogenesis of fibrosis occurring during the last phase of chronic TNBS colitis, it is first necessary to discuss our previous studies of IL-13 and its relation to the induction of TGF-β_1 and collagen deposition. In these studies, we showed that IL-13 induces production of TGF-β_1 via a two-stage process involving 1) the induction of the cell surface IL-13Rα_2 receptor by the combined activity of TNF-α and either IL-4 or IL-13 and 2) IL-13 signaling through this receptor and activation of the TGF-β_1 promoter via AP-1 (6). The first stage involves signaling by the IL-13Rα_1 receptor to transduce a Stat6 signal and, concomitantly, signaling by the TNF-α receptor to transduce a NF-κB signal that together activate the promoter of IL-13Rα_2 and thus cause up-regulation of the latter and its greatly increased expression on the cell membrane. Of interest, this receptor was formerly considered to be a nonsignaling receptor that functioned exclusively as a decoy receptor and that diminished rather than facilitated IL-13 signaling (25, 26, 50). The second stage involves generation of an AP-1 signal composed of c-jun and Fra-2 that acts on the TGF-β_1 promoter by itself or in concert with as yet unknown factors to cause activation of the promoter and TGF-β_1 production. This two-stage process has been verified in two models of inflammation, oxazolone colitis and bleomycin-induced pulmonary fibrosis, in which it was shown that various ways of inhibiting IL-13Rα_2 expression or its signaling function reduces TGF-β_1 production and fibrosis (6).

In the light of these earlier studies, we developed the hypothesis that in the chronic TNBS colitis model studied here, the induction of IL-13 production, in the context of an inflammation that is also characterized by the production of TNF-α, leads to increasing expression of the IL-13Rα_2 receptor on monocytes and macrophages and then IL-13 signaling via this receptor to obtain TGF-β_1 secretion and its downstream consequence, collagen synthesis. It should be noted that we could entertain this hypothesis because we found that Smad7, an inhibitory Smad induced by TGF-β_1 in the early stage of chronic TNBS colitis, is not expressed during the late stage of the colitis when TGF-β_1 secretion occurs; thus, TGF-β_1 signaling during this period is not impeded by this inhibitory factor (34, 51–53).

In studies to test the validity of this hypothesis, we administered agents that blocked IL-13 interaction with IL-13Rα_2 or down-regulated expression of this receptor; in addition, we blocked TGF-β_1 interaction with its receptor and thus TGF-β_1 activation of Smads. Blockade of IL-13 was accomplished by intranasal administration of a plasmid encoding soluble IL-13Rα_2-Fc (pCI-sIL-13Rα_2) that leads to in vivo production of a soluble receptor fusion protein; the latter, in turn, binds IL-13 with high affinity and interferes with its ability to bind to the membrane-bound receptor (54). Down-regulation of IL-13Rα_2 expression, in contrast, was achieved by the administration of IL-13Rα_2-specific siRNA encapsulated in a viral envelope (HVJ-E) that allows highly efficient delivery of siRNA to cells in vivo. Finally, we blocked TGF-β_1 signaling very simply by the administration of anti-TGF-β_1-Ab. The treatment of the mice with these agents was initiated on day 35 following initiation of the chronic colitis when IL-13 and TGF-β_1 expression was initially noted, but collagen deposition in the gut could not yet be observed.

As expected, whereas blockade of IL-13 with a receptor-fusion protein did not influence the expression of the IL-13Rα_1, it greatly down-regulated both the expression of the IL-13Rα_2 and its signaling, the latter being indicated by the concomitant down-regulation of c-jun; in addition, it inhibited TGF-β_1 production and fibrosis. This result strongly suggested but did not prove that signaling via IL-13Rα_2 is necessary for TGF-β_1 induction and fibrosis. Such proof was obtained, however, with the finding that down-regulation of the expression of IL-13Rα_2 also resulted in abrogation of TGF-β_1 induction and fibrosis. Finally, the blockade of TGF-β_1 with anti-TGF-β_1 at a point in the pathway downstream of IL-13Rα_1 led to down-regulation of the expression of activated Smad3 and decreased collagen deposition, indicating that fibrosis in this model was in fact mediated by TGF-β_1. Thus, the observed effects of the inhibition of IL-13 and TGF-β_1 signaling fully validate the concept established previously that IL-13 production in an inflammation induces fibrosis via a two-stage process involving

first the induction of IL-13Rα_2 and the signaling via this receptor for the induction of TGF-β_1.

Given the unique pattern of cytokines that characterizes the chronic TNBS colitis model studied here, it is reasonable to ask about the relevance of this model to the development of fibrosis in CD. First, as alluded to above, the sequential Th1/Th17 cytokine pattern may mirror different phases of mucosal inflammation that are separate in murine models but are concomitant in human disease. Second, whereas CD is a Th1/Th17-driven inflammation, it could nevertheless lead to the development of cells that produce IL-13, the key profibrotic cytokine identified in this study. This theory would fit with the idea that the cells producing IL-13 are not IL-13-producing Th2 T cells, but rather APCs. In this context, although it is clear that T cells in Crohn's lesions do not produce impressive amounts of TGF-β_1 (55, 56), other cells in these lesions have not yet been studied to determine their potential to produce this cytokine. Finally, if indeed the pathogenesis of fibrosis in the model studied here can be applied to CD, then one can predict that the inflammatory process and the fibrotic process are distinct in this disease and thus are amenable to separate therapeutic intervention. Of particular importance in this latter context is the identification of a heretofore unheralded receptor, the cell surface IL-13Rα_2 receptor, as the nodal point of the fibrosis. Thus, therapies directed at the function of this receptor offer a new approach to the treatment of fibrosis CD.

Disclosures

The authors have no financial conflict of interest.

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