GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

M2 Macrophages and Phenotypic Modulation of Intestinal Smooth Muscle Cells Characterize Inflammatory Stricture Formation in Rats

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The progression of Crohn disease to intestinal stricture formation is poorly controlled, and the pathogenesis is unclear, although increased smooth muscle mass is present. A previously described rat model of trinitrobenzenesulfonic acid–induced colitis is re-examined here. Although inflammation of the mid-descending colon typically resolved, a subset showed characteristic stricturing by day 16, with an inflammatory infiltrate in the neuromuscular layers including eosinophils, CD3-positive T cells, and CD68-positive macrophages. Closer study identified CD163-positive, CD206-positive, and arginase-positive cells, indicating a M2 macrophage phenotype. Stricturing involved ongoing proliferation of intestinal smooth muscle cells (ISMC) with expression of platelet-derived growth factor receptor beta and progressive loss of phenotypic markers, and stable expression of hypoxia inducible factor 1 subunit alpha. In parallel, collagen I and III showed a selective and progressive increase over time. A culture model of the stricture phenotype of ISMC showed stable hypoxia inducible factor 1 subunit alpha expression that promoted growth and improved both survival and growth in models of experimental ischemia. This phenotype was hyperproliferative to serum and platelet-derived growth factor BB, and unresponsive to transforming growth factor beta, a prominent cytokine of M2 macrophages, compared with control ISMC. We identified a hyperplastic phenotype of ISMC, uniquely adapted to an ischemic environment to drive smooth muscle layer expansion, which may reveal new targets for treating intestinal fibrosis. (*Am J Pathol* 2020, 190: 1843–1858; <https://doi.org/10.1016/j.ajpath.2020.05.015>)

Crohn disease (CD) is an inflammatory bowel disease causing chronic transmural inflammation that typically affects the terminal ileum and colon.¹ By the time of diagnosis, roughly a quarter of patients already have complications such as stenosis or abscess formation, and nearly all will do so after a decade.^{2,3} This progression to stricture formation and subsequent surgery in CD is only poorly influenced by current therapy. By contrast, the other principal form of inflammatory bowel disease, ulcerative colitis, also causes an incurable, relapsing, chronic inflammation, but this affects the colonic mucosa and only rarely affects the deeper structures to any extent and is not associated with stricture formation.⁴ This implies that the pathogenesis of CD involves key alterations or participation of the deeper neuromuscular layer, which may be related to the high frequency of recurrent stricture formation following surgery.^{5,6}

Animal models have been valuable in illustrating the processes that may occur in CD, identifying inflammatory processes and allowing interventions that clarify the underlying molecular and cellular biology. For example, the widely used model of trinitrobenzenesulfonic acid (TNBS)-induced inflammation in rodents causes a TH1-dominant transmural inflammation that resembles aspects of CD.⁷ Key findings from this model that are relevant to understanding the pathogenesis of stricture formation include the demonstration that inflammation causes proliferation of the intestinal smooth muscle cells (ISMC), which then leads to a lasting thickening of the intestinal wall.^{8–10} This process begins at the onset of inflammation, when proinflammatory cytokines induce expression of platelet-derived growth factor (PDGF)-R β in ISMC *in vitro* and *in vivo* that confers

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sensitivity to the major mesenchymal mitogen PDGF-BB.^{11,12} Further, the phenotype of the ISMC remains altered even after proliferation ceases and inflammation is resolved, with decreased expression of smooth muscle marker proteins.¹⁰ Recent work showed that protracted proliferation of rat ISMC *in vitro*, as might occur in chronic disease *in vivo*, caused epigenetic modification of phenotype with outcomes similar to those identified in ISMC from Crohn strictures, but absent from adjacent normal regions.¹³

Although intestinal strictures in CD have been characterized as fibrostenotic, implying a noncellular accumulation of collagen, there is increasing evidence that increased smooth muscle, with an altered ISMC phenotype, is the predominant component.^{13–16} ISMC in the Crohn stricture are typically labeled with antibodies to markers for the contractile smooth muscle phenotype, such as α -smooth muscle actin (SMA), SM-22, or CG4A, which detects cells expressing both α - and γ -smooth muscle actin. However, coexpression with vimentin was also detected in some cells, indicative of a dedifferentiated smooth muscle phenotype.¹⁷ The key role of ISMC is strongly supported in a recent quantitative study of Crohn strictures, showing that expansion of the smooth muscle layers is the single major contributor to the outcome, with fibrosis (ie, inappropriate expression of extracellular matrix²) serving only a minimal role.¹⁶

This directly implicates the ISMC in the development of the stricture and the resulting luminal narrowing. Although thickening of the inflamed intestinal wall is well-reported, the stricturing process has been difficult to recapitulate in animal models. For example, smooth muscle hyperplasia and hypertrophy occur in TNBS colitis in both rats and mice, but this is normally observed for a period of a few days at most, when inflammation resolves and normal intestinal function returns. However, in Sprague-Dawley rats followed for a longer period (30 to 90 days), it was found that a subset of animals developed strictures at the site of previous inflammation.¹⁸ By 30 days post-TNBS, about 30% of animals showed stenotic intestinal tissue with marked thickening and disorganization of the smooth muscle layers, which persisted to at least 90 days post-TNBS. Microscopic analysis showed normal tissue surrounding the strictured region, which displayed aberrant mucosal architecture in all samples. The ISMC showed an altered phenotype characterized by reduced desmin and α -SMA labeling, and the onset of vimentin expression.

This model mimics the sporadic stricture formation seen in CD. Because it occurred after a Crohn-like inflammation, it could offer valuable insight into this poorly understood phenomenon. Accordingly, this model was further examined to identify earlier events that might predict or define stricture pathogenesis. This study identified and characterized divergent responses among animals as the acute colitis was resolved, identifying a persistent inflammatory milieu with the presence of M2 (alternatively activated) macrophages. Proliferating ISMC displayed abundant PDGF-R β expression that was associated with reduced expression of contractile

markers, evidence for loss of contractile phenotype. However, the authors also found prominent nuclear HIF-1 α localization that correlated with increased survival during metabolic or ischemic challenge *in vitro*. Further, the tissue culture model of the stricture phenotype of ISMC showed insensitivity to TGF β , a cytokine that normally strongly suppressed ISMC growth. This suggested that in some animals, ISMC undergo ongoing exposure to PDGF-BB and inflammatory cytokines, leading to a permanently dedifferentiated phenotype that drives stricture formation. These histologic and immunocytochemical changes can provide insight into the development of approaches that can minimize or reverse the damaging effects of stricturing in CD.

Materials and Methods

TNBS Colitis

Adult Sprague-Dawley rats were obtained from Charles River Laboratories (Montreal, QC, Canada) and housed in pairs in microfilter-isolated cages with free access to food and water, for at least 5 days prior to use. To induce inflammation, 500 μ L of 200 mmol/L TNBS (Fluka; Canadian Life Science, Peterborough, ON, Canada) dissolved in 50% ethanol was instilled into the mid-descending colon of rats (200 to 250 g) under light anesthesia with inhaled isoflurane, as previously described.¹¹ Animals received subcutaneous injections with 1 mL of lactated Ringer saline from days 1 to 4 post-TNBS. There was no animal mortality associated with this procedure, and all experimental procedures were approved by the Queen's University Animal Care Committee.

Tissue Preparation

At day 6 (D6), 16 (D16), 35 (D35), or 90 (D90) post-TNBS administration, rats were sacrificed by cervical dislocation under isoflurane anesthesia. The mid-descending colon was removed and fixed immediately in 4% neutral buffered formalin or opened along the mesenteric border and pinned flat in phosphate-buffered saline (PBS; pH 7.4; NaCl 8 g, KCl 0.2 g, Na₂HPO₄ 1.44 g, KH₂PO₄ 0.24 g, double-distilled H₂O 1 L) before fixing for 24 hours. Fixed tissue was embedded in paraffin and sectioned at 4 μ m.

Histology and Immunohistochemistry

Sections were stained with hematoxylin and eosin for routine examination as described previously.¹⁸ Consecutive sections were treated with Sirius Red/Fast Green (BDH Chemicals; VVR International, Mississauga, ON, Canada) differential stain to visualize the proportion of collagen to cellular protein as described.¹⁸ Images of stained tissue were acquired by light microscopy (Olympus BX60; Infinity 2 Camera; Infinity Capture; Teledyne Lumenera Corp., Ottawa, ON, Canada), and the proportions of collagen (red) and noncollagen (green) protein were determined using

Table 1 Primary and Secondary Antibodies Used for Immunocytochemistry

Antibody	Source	Catalog number	Dilution	Antigen retrieval
Primary antibody				
Arginase-1	NEB	93668	1:200	DAKO TRS
CD3	DAKO	N1580	Pre-diluted	DAKO TRS
CD68	Serotec	MCA341GA clone ED1	1:300	DAKO TRS
CD163	Serotec	MCA342GA clone ED2	1:300	DAKO TRS
CD206	Santa Cruz	sc-58986	1:100	NA
Cleaved caspase-3	NEB	9664	1:200	Citrate, pH6
Collagen I	Novus	NB600-408	1:150	DAKO TRS
Collagen III	Novus	NB600-594	1:150	Citrate, pH 6
HIF 1 α	Novus	NB100-479	1:200	DAKO TRS
iNOS	Novus	NB300-605	1:200	DAKO TRS
iNOS	BD Transduction	610332	1:500	DAKO TRS
Mast cell tryptase	DAKO	M7052	1:200	DAKO TRS
PCNA	DAKO	2586	1:4000	NA
PDGF-R β	NEB	3169	1:200	DAKO TRS
α -SM22	Abcam	ab14106	1:2000	NA
Smoothelin	Abcam	ab219652	1:2000	DAKO TRS
Secondary antibody				
Goat anti-mouse Alexa 488	Life Technologies	A11001	1:1000	NA
Goat anti-mouse Alexa 555	Life Technologies	A21422	1:1000	NA
Goat anti-rabbit Alexa 555	Life Technologies	A21428	1:2000	NA
Goat anti-rabbit Alexa 488	Life Technologies	A11008	1:2000	NA

HIF 1 α , hypoxia-inducible factor 1-alpha; iNOS, inducible nitric oxide synthase; NA, not applicable; PCNA, proliferating cell nuclear antigen; PDGF-R β platelet derived growth factor receptor beta.

Image Pro Plus software version 6 (Media Cybernetics, Rockville, MD); at least three nonadjacent images were analyzed for each tissue.

Specific cell types and cell components were detected using immunocytochemistry. Smooth muscle cells were detected with anti- α -SM22 or anti-smoothelin antibodies (both 1:2000; Abcam, Cambridge, MA). The presence of macrophages was detected using anti-CD68 (1:150; AbD Serotec, Kidlington, UK), anti-CD163 (1:150; AbD Serotec), anti-CD206 (1:100; Santa Cruz Biotechnology, Dallas, TX), or anti-arginase (1:200; Cell Signaling Technology, Danvers, MA), and T cells were detected using anti-pan T antibodies (anti-CD3, pre-diluted; Dako Canada, Mississauga, ON, Canada). Proliferating cells were detected with antibodies to proliferating cell nuclear antigen (PCNA; 1:4000; Cell Signaling Technology). Apoptotic cells were detected with anti-cleaved caspase-3 antibodies (1:200; Cell Signaling Technology). The receptor for PDGF-BB was detected using anti-PDGFR β antibodies (Cell Signaling Technology; 1:200), and hypoxia was detected with anti-HIF1 α antibodies (1:200; Novus, Oakville, ON, Canada). Collagen was detected with anti-collagen I and anti-collagen III (both 1:150; Novus). Further details are provided in [Table 1](#).

All antibodies were diluted in PBS with 0.2% Tween-20 (PBS-T), and where necessary, tissue was incubated with citrate buffer (pH 6) at 95°C for 20 minutes or with 1 $\times$ target retrieval solution (TRS; Dako Canada) according to the manufacturer's directions. Slides were then incubated with 1% goat serum in PBS-T for 1 hour to block nonspecific

binding before overnight incubation in primary antibodies at 4°C, followed by incubation in the appropriate Alexa-linked secondary antibodies (Millipore Canada, Etobicoke, ON, Canada) for 2 hours at room temperature. Hoechst 33342 (Sigma-Aldrich, St. Louis, MO; 1 μ g/mL) was used to label all nuclei, and staining was visualized by fluorescence microscopy (Olympus BX51; Olympus, Tokyo, Japan).

Images of three to six nonadjacent fields of were acquired using a fluorescence microscope (Olympus BX51) and Retiga2000R camera linked to Q-Capture Pro 7 (both Teddyne QImaging, Surrey, BC, Canada). Images of nonadjacent regions to the stricture were acquired in areas at least 500 μ m distant from affected regions.

For measurement of the intensity of smooth muscle marker labeling, images were acquired at standardized nonsaturating exposure times. Each glass slide contained both control sections (noninflamed; no prior TNBS exposure) as well as experimental sections to allow internal comparisons. The intensity of immunostaining was measured for 10 nonadjacent circular smooth muscle (CSM) cells per image (Image Pro Plus) for at least 3 nonadjacent images per section. These were averaged and expressed relative to control ISMC intensity.

Cell Culture

Primary cultures of adult rat colonic CSM cells were obtained from strips of circular muscle that were microdissected from the mid-descending colon. These were

Figure 1 The developing stricture is detectable in some rats at day 16 (D16) post-trinitrobenzenesulfonic acid (TNBS) instillation. **A** and **B**: Representative low-power micrographs of longitudinal sections showing stricture formation in rats at D16 post-TNBS instillation, with arrows indicating proximal and distal margins. **C**: Macroscopic appearance of stricture in colon opened along the mesenteric border. Arrows indicate the margins of the stricture. **D–I**: Histology of divergent responses after colitis, showing recovery versus stricturing outcomes. Representative images at the same magnification of longitudinal sections of control (**D**), day 6 (**E**), and day 16 (**F**) post-colitis show normal recovery, compared with a developing stricture at day 16 (**G**). By day 35, animals typically recovered, showing normal histology with thickened smooth muscle (**H**), whereas others showed stricture formation (**I**). **J**: Weights of animals during and after TNBS colitis show a lag in weight recovery between animals that formed intestinal strictures versus those that did not, an outcome that is significant on days 7 and 10. $n = 13$ to 22 animals on days 7 and 10 (**J**); $n = 21$ to 27 animals on days 0 to 4 (**J**); $n = 5$ to 17 animals on days 16 and 35 (**J**). $*P < 0.05$ versus time-matched cohorts. Scale bars: 500 μ m (**A** and **B**); 2 mm (**C**); 50 μ m (**D** to **G**); 100 μ m (**H** and **I**). CSM, circular smooth muscle; MM, muscularis mucosa.

incubated in HEPES-buffered digestion solution [papain (0.5 mg/mL), bovine serum albumin (1 mg/mL), dithiothreitol (1 μ mol/L), collagenase (type F, 0.25 mg/mL)] at 4°C for 2 hours, followed by 1 hour at room temperature and then 37°C for 1 hour. Individual cells were obtained by trituration and these were plated onto 60-mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS; Invitrogen, Carlsbad, CA). Culture purity was confirmed by positive staining for smooth muscle markers and the lack of immunostaining for glia or interstitial cells of Cajal as described previously.¹⁰ Once cultures reached 80% to 95% confluency, they were passaged (1:2 ratio) 1 to 3 times to generate low-passage cells, or 10 or more times to generate high-passage cells.¹³

For antibody staining of cultured ISMC, cells were plated on collagen-coated glass cover slips (Sigma-Aldrich) and incubated in DMEM containing 5% FCS for 48 hours. Cells were fixed in neutral buffered formalin and incubated with antibodies to HIF-1 α , and staining was visualized as above.

For experimental treatments, ISMC were plated into 96-well microplates at 7500 cells/well in DMEM with 5% FCS for 24 hours, serum-deprived for 24 hours, and exposed to the metabolic uncoupler dinitrophenol (50 to 750 μ mol/L in DMEM). Alternatively, cells were incubated in a hypoxic chamber (1% O₂; BioSpherix, Parish, NY) for 24 hours. The effect of inhibition or activation of HIF-1 α on cell proliferation was tested using chetomin or cobalt chloride (Sigma-Aldrich). In some experiments, cells were treated with PDGF-BB (0.05 to 50 ng/mL; Sigma-Aldrich) and cotreatment or 1-hour pretreatment with TGF- β (50 ng/mL). Cell number was assessed using the WST-8 assay (Cayman, Ann Arbor, MI) according to the manufacturer's instructions.

Western Blot Analysis

Cells were treated for 30 minutes or 18 hours with TGF β (50 ng/mL), then lysed using 1 $\times$ Laemmli sample buffer (20 mmol/L Tris/HCl, 6% glycerol, 0.5% SDS, 2% β -mercaptoethanol, 0.001% bromophenol blue). Samples were resolved using 10% SDS-PAGE, then transferred to polyvinylidene difluoride membrane using a semidry apparatus (Bio-Rad, Hercules, CA). Membranes were incubated in blocking buffer (5% skim milk in Tris-buffered saline with 0.2% Tween 20) for 60 minutes at room temperature. Blots were incubated at 4°C overnight with primary anti-pSMAD 2/3 (1:1000; Novus), then horseradish peroxidase-linked secondary antibodies for 2 hours at room temperature and visualized using a chemiluminescent substrate (Luminata Forte; Millipore Canada) with a ChemiDoc MP system (Bio-Rad). Blots were reprobed with anti- β -actin antibodies (1:5000; Sigma-Aldrich) to confirm equal loading of cell samples.

Statistics

Quantitative data are expressed as means $\pm$ SEM of n animals. Differences between control and treatment conditions

were considered significant for $P \leq 0.05$ using analysis of variance or Kruskal-Wallis with Dunn's post-test. Differences between two groups are considered significant for $P \leq 0.05$ using a two-tailed t -test.

Results

Sporadic Incipient Stricture Formation in Rats at D16 Post-TNBS

The authors' earlier study of the outcome of TNBS-induced colitis in Sprague-Dawley rats showed that a subset of animals developed intestinal strictures by 35 days post-TNBS (D35), with characteristics similar to those of human strictures in CD.¹⁸ Now, this was examined more closely to identify the events during the progression of inflammation that might correlate with subsequent stricture formation.

Application of TNBS causes a transmural inflammation that is predictably situated in the mid-descending colon. The peak inflammatory response on day 6 subsequently resolves, and the majority of animals display only a thickened muscular wall by D35.^{9,10} Standard histology showed a typical severe transmural inflammation by D6, with no apparent differences among animals by qualitative examination (Figure 1, D and E). By D16, roughly halfway to the previous endpoint of D35, the mid-descending colon appeared similar to the control. Indeed, the histology of the mid-descending colon of most animals showed an intact mucosa and no evidence of inflammation, although the smooth muscle layers were noticeably thickened (Figure 1F) as described.⁹ However, a subset of rats (25/102) displayed ongoing circumferential inflammation and obvious changes in tissue microarchitecture. There was prominent local thickening of the smooth muscle layers (Figure 1, A–C), with mucosal damage and variable cellular disorganization in the muscularis externa accompanied by an immune cell infiltrate (Figure 1G). Overall, this resembled the stricture seen at D35, where both macroscopic and microscopic changes were readily apparent (Figure 1, G–I).

Figure 2 Although inflammation typically resolves, there is a persistent presence of immune cells in the smooth muscle in developing strictures. **A–C:** Absence of immune cell infiltration in circular smooth muscle layer of rat colon in day 16 (D16) recovery animals (**A**, left panel) versus continued, prominent presence in developing strictures (**A**, right panel; arrows indicate eosinophils). Quantification of eosinophils among smooth muscle cells showing large numbers on day 6 post-trinitrobenzenesulfonic acid (TNBS) instillation, which remained detectable in strictured tissue by D90 post-TNBS (**B**). Representative images of tissue from D16 stricture labeled with anti-CD68 (left) or anti-CD3 (right) antibodies (arrows) showing the presence of infiltrating macrophages and T cells, respectively (**C**). **D** and **E:** Quantification of CD68-positive (**D**) and CD3-positive (**E**) cells in the circular smooth muscle layer of animals during the time course of stricture formation, showing persistence in stricture formation versus low levels in recovery animals. * $P < 0.05$ versus time-matched cohorts. Scale bars = 20 μm (**A** and **C**).

Figure 3 The smooth muscle region of developing strictures acquires and maintains an M2 macrophage phenotype, whereas an M1 phenotype persists in the overlying mucosa. **A:** Immunostaining for inducible nitric oxide synthase (iNOS; green) in a day 16 (D16) developing stricture, showing strong positive staining only in the mucosal edge of the developing stricture, which was absent from the adjacent nonstrictured region. Image representative of all developing strictures; **dashed line** indicates stricture. **B:** Image showing staining with antibodies to CD163 (red), typically expressed in M2 and resident macrophage phenotypes, in the circular smooth muscle (**arrows**) of a D16 developing stricture. **C:** Quantification of staining for CD163 in the smooth muscle of developing and established strictures. **D:** Image of immunostaining of macrophages for CD206 (red), an M2 phenotype marker, in the smooth muscle of a stricture at D16 [note pleomorphic features (**arrows**)]. **E:** Quantification of CD206-positive cells in the smooth muscle of strictures on D16 and D35. **F–H:** Representative images of immunostaining for arginase, a definitive marker for the M2 phenotype of macrophages in a D16 developing stricture. **F: Left panel:** Low-power images of arginase staining that is absent from the mucosa (MU) but abundant in the underlying smooth muscle (SM). **Right panel:** Matching image of nuclei stained with H333258. **G:** Higher magnification image of arginase-positive cells (**arrows**) in the smooth muscle region. **H:** Quantification of arginase staining in the smooth muscle of developing and established strictures. * $P < 0.05$ versus time matched recovery. Scale bars: 75 μm (**A** and **F**); 30 μm (**B**); 20 μm (**D**); 40 μm (**G**). nd, not done. TNBS, trinitrobenzenesulfonic acid.

Examination of the course of weight loss and recovery during TNBS-induced colitis showed a similar initial weight loss among animals regardless of stricturing outcome. However, there was a significantly slower weight gain during the recovery phase for animals that went on to show stricture formation (Figure 1J) ($n = 13$ to 22 rats/time point). Combined with the histological outcome, this shows that progressive changes leading to stricture formation are present well before D35.

Inflammatory Cells Persist in the Developing Stricture

In the subset of animals with persistent inflammation, there was a characteristic abundance of immune cells in the submucosa and smooth muscle of the mid-descending colon (Figure 1), and the major contributing cell types were quantified. Evaluation of sections stained with hematoxylin and eosin showed that the number of eosinophils in the smooth muscle was greatly increased in D6 animals but remained equally high in those showing continued inflammation at D16 (D16 stricture) (Figure 2A). By contrast, eosinophils became rare or absent in cohort animals that were recovering normally (D16

recovery) (Figure 2A). Similarly, eosinophils were typically present in the smooth muscle of D35 strictures, whereas they were absent from D35 recovered cohorts, and this persisted through D90 (Figure 2B).

In the smooth muscle layers, immunocytochemistry with antibodies to the pan-macrophage marker CD68 or the T-cell marker CD3 (CD4/8 T cells) showed that both markers were nearly completely absent from the control intestine, but readily detected in the inflamed intestine on D6 (Figure 2C). These dropped to undetectable levels in D16 recovery animals, but remained high in D16 stricture animals, with a similar pattern evident on D35 and D90 (Figure 2, D and E).

Mast cells were normally undetectable in the smooth muscle layers of the control or inflamed (D6) intestine, or at the site of prior inflammation in D16 recovery animals. However, they were reliably present in areas adjacent to stricturing (>100 to $300 \mu\text{m}$ distant from stricture margin and underlying intact mucosa), at 11 ± 2 (3) on D16, 9 ± 2 (5) on D35 and 12 ± 2 (3) on D90 [mast cells/ $10^5 \mu\text{m}^2 \pm \text{SEM}$ (n animals)]. This did not correlate with the presence of mast cells in the stricture, which was detected in only 2 of 5 D16 rats, 1 of 5 rats at D35, and 0 of 3 rats at D90.

Figure 4 Decreased expression of smooth muscle marker proteins in colitis is markedly worsened in developing and established strictures. **A:** Image of control colon labeled with antibodies to SM22. **B and C:** Images of sections of circular smooth muscle at day 16 (D16) and D35, comparing staining for SM22 in recovery (**top panels**) versus stricture (**bottom panels**) animals. **D:** Quantification of SM22 immunoreactivity over the time course of resolution or stricture formation. The intensity of SM22 labeling per smooth muscle cell decreases by D6 post-trinitrobenzenesulfonic acid (TNBS) instillation and is maintained thereafter in recovery tissues. However, this further decreases in stricture formation. **E:** Parallel decline in expression of smoothelin, a second marker of smooth muscle phenotype, in D16 recovery animals versus control, with a further significant decrease in the D16 developing stricture. $n = 3$ to 7 animals per time point (**D** and **E**). * $P < 0.05$ versus control; † $P < 0.05$ versus time-matched cohorts (analysis of variance); ‡ $P < 0.05$ versus control or recovery. Scale bars: 10 μm (**A**); 20 μm (**B** and **C**). CSM, circular smooth muscle; MM, muscularis mucosa.

The Presence of M2 Macrophages Characterize the Developing Stricture

The macrophages in the developing stricture were then further assessed for M1 versus M2 (alternatively activated) phenotypes. In D16 developing strictures, inducible nitric oxide synthase (iNOS)-positive cells were prominently present at the mucosal verge, whereas they were absent from adjacent regions with intact mucosal structure (Figure 3A). These cells showed no overlap with CD68 (not shown) and are evidence for ongoing inflammation. Immunoreactivity for CD163, a general marker for the M2 phenotype,¹⁹ was distributed among the smooth muscle cells (Figure 3, B and C), as was staining for CD206, also considered to be a marker for the M2 phenotype (Figure 3, D and E).²⁰ However, CD163 is also present on resident macrophages in the control intestine,²¹ and CD206 similarly overlaps with this population.²² Therefore, staining for arginase was used for definitive identification.²³ The characteristic staining of developing strictures on D16, with a prominent presence of arginase-positive macrophages distributed in graded fashion in the submucosa and smooth muscle, continued on D35 (Figure 3, F–H). There was no overlap of staining for iNOS or CD163, and quantitative analysis showed that both CD163 and arginase staining were selectively present in the developing and established strictures (Figure 3).

Overall, the large numbers of immune cells in developing and established strictures was distinct from their recovered counterparts and show the presence of continuing inflammation, with aspects of both an acute and chronic nature. Further, the appearance and persistence of arginase staining by D16 is evidence for a M2 phenotype of macrophages in the deeper layers.

Progressive Phenotypic Changes in Smooth Muscle during Stricture Formation

In earlier work, the authors showed that inflammatory factors caused proliferation of the ISMC and an altered cellular phenotype,^{10,11} raising the possibility that protracted inflammation leads to the locally severe alterations in ISMC number and nature that typify the stricture.¹³ To identify this process at an intermediate step, this was assessed in D16 rats, using image analysis of outcomes of immunocytochemistry for the smooth muscle marker protein SM22, a calponin-related protein that is expressed specifically in smooth muscle *in vivo*.²⁴

In control tissues, staining for SM22 was both strong and selective among the smooth muscle layers, whereas it appeared markedly reduced following colitis (Figure 4, A–C). Image analysis confirmed this, showing that staining intensity was reduced sharply by D6 post-TNBS to $54 \pm 3\%$ of control ($n = 4$ to 7) with no significant subsequent

Figure 5 The relative proportion of collagen, especially collagen III, increases during stricture formation, although extracellular matrix remains a small component. Sections were stained with Sirius Red/Fast Green to detect collagen (red) versus non-collagen protein (green), or with antibodies to collagen I or III. **A–C:** Images of histochemistry showing the distribution of collagen surrounding the smooth muscle cells in control (**A**), day 16 (D16) recovery (**B**), and D16 stricture (**C**). Note the increase in smooth muscle size due to hypertrophy (**B**) and loss of distinct pericellular collagen outlines (**C**). **D:** Analysis of proportion of collagen showing a progressive increase with stricture formation, with its proportion at D35 more than double that of control. Nonetheless, the great majority of protein in the strictured region is noncollagen, and therefore, cellular. **E** and **F:** Examples of up-regulation of collagen I (**E**) and collagen III (**F**) expression in tissue sections visualized by fluorescence microscopy. **G** and **H:** Image analysis of collagen staining, showing no change in collagen I staining in D6 or D16 recovery tissue, with significant increases in D16 and D35 strictures (**G**). Collagen III staining (**H**) shows a transient increase by day 6 post-trinitrobenzenesulfonic acid (TNBS) instillation that was reversed by D16 recovery, whereas cumulative large increases arose in D16 and D35 strictures. * $P < 0.05$ versus control (analysis of variance). Scale bars: 20 μm (**A–C**, **E**, and **F**).

reversal among either D16 recovery animals or nonstrictured D35 animals (Figure 4D). However, there was a significant further decrease in staining seen in inflamed versus noninflamed regions by D16, and similarly, in the strictured region versus nonstrictured cohort tissues on D35 (Figure 4D).

Immunocytochemistry was used for smoothelin, an additional specific marker of smooth muscle phenotype,²⁵ and found a similar decrease in expression between control and D16 recovery tissues and a further significant decrease between D16 recovery versus D16 stricture (Figure 4E). These changes were specific to the strictured regions and were absent from adjacent regions, which macroscopically and microscopically resembled those of recovery animals (data not shown). This is interpreted to show that inflammation causes a lasting decrease in expression of smooth muscle markers and further, that

protracted inflammation causes progressive further loss that is associated with the stricture phenotype.

Increased Collagen Expression in Strictured Tissue

Although the stricture is largely a cellular outcome in both humans and animal models,^{14,16,18} there are reports of increased collagen in human strictures²⁶ and in animal models.^{18,27} Therefore, the progression of collagen deposition during stricture formation in the TNBS model was determined by analyzing the proportion of collagen to noncollagenous (cytosolic or cellular) protein in histological sections using the Sirius Red/Fast Green method as reported earlier.¹⁸ For this, the proportions of red-stained collagen and green-stained noncollagen protein in the CSM layers of control tissue were measured to establish baseline levels in this region. In control animals, collagen formed a distinct

outline around the smooth muscle cells, occupying $15.7 \pm 1.1\%$ ($n = 3$) of total protein (Figure 5, A–D). Although this showed no significant change during the progression from control through D6 to D16 recovery, D16 stricture tissue showed a disorganized, more diffuse staining pattern with a significant increase in the proportion of collagen that was further increased in D35 strictures to nearly a twofold increase over control (Figure 5D).

Progressive collagen deposition over the course of stricture formation was examined further by evaluation of the distribution of collagen I and III, the major subtypes expressed in the human and rat intestine.^{28,29} In control colon, immunohistochemistry showed the presence of collagen I in the intercellular regions around ISMC, with strong expression in the areas surrounding the myenteric plexus and along blood

vessels; by contrast, staining of collagen I in the D16 strictures was much less uniform, with areas of locally dense labeling in the smooth muscle layer (Figure 5E). This pattern remained present in D35 strictures, along with a significant increase in the relative area of collagen I staining by 1.7-fold relative to control ($P < 0.05$, $n = 5$) (Figure 5G).

Staining for collagen III showed a similar pattern in the control CSM layer, although the relative area of labeling was significantly less than for collagen I (Figure 5F). There was an increase by D6 post-TNBS, but the most striking change was the subsequent strong and progressive increase in D16 and D35 strictures, reaching almost ninefold more than control levels ($P < 0.05$ for each) (Figure 5H).

It is concluded that these methods converge in identifying an increase in the presence of extracellular collagen in both developing and established strictures. This outcome is distinct from that of resolved inflammation, where there was no significant increase, and shows that the alterations to the phenotype of the ISMC in stricture formation include an increase in expression of extracellular matrix.

ISMC Proliferation and Apoptosis in the Stricture

In TNBS-induced inflammation, smooth muscle proliferation early in the time course causes an increased intestinal wall thickness by day 6.⁹ The smooth muscle cells then revert to a quiescent phenotype, although the increased wall thickness is permanent and the ISMC maintain heightened sensitivity to further proliferative stimuli.¹² Therefore, chronic inflammation could support a continually proliferative smooth muscle phenotype that supports stricture formation. This was examined in sections of mid-descending colon using immunocytochemistry for the proliferation marker PCNA, a nuclear marker of cells in the S phase of cell division,³⁰ with colabeling for SM22 to verify smooth muscle cell identity.

Figure 6 Both proliferation and apoptotic death of smooth muscle cells characterize stricture formation. **A:** Representative images of sections of colon showing detection of smooth muscle proliferation with anti-proliferating cell nuclear antigen (PCNA) antibodies (red). **Left panel:** Sections of day 16 (D16) post-trinitrobenzenesulfonic acid (TNBS) recovery labeled with anti-PCNA (green) and anti-SM22 (red) antibodies showing abundant PCNA-positive cells in the epithelial crypts (asterisks), which are absent from the underlying smooth muscle layers. Nuclei are stained with Hoechst (blue). **Right panel:** Image showing example of PCNA colocalization with SM22 (arrows) in the smooth muscle layer of a section of strictured colon at D16. **B:** Quantification of PCNA-labeled intestinal smooth muscle cells (ISMC) during acute colitis (D6), and in recovered or strictured tissue showing persistently increased labeling in stricture formation, with no labeling detected in D35 or D90 recovery. **C:** Images of apoptosis detected by immunostaining for cleaved caspase-3 (CC3). **Left panel:** Positive expression of CC3 in intact epithelium (red; asterisks) in a region adjacent to stricture. **Right panel:** Example of colocalization of CC3 with smooth muscle cells using anti- α -smooth muscle actin, in D16 developing stricture (green; arrows). **D:** Quantification of colocalization of CC3 and SMA in smooth muscle cells during colitis and recovery or stricture formation. Although not detectable after D6 in recovery animals, this remained increased with stricture formation. * $P < 0.05$ versus control (Kruskal Wallance analysis of variance). Scale bars: 20 μ m (A and C).

Figure 7 Platelet-derived growth factor receptor beta (PDGFR-β) is expressed in smooth muscle in colitis and resolves with recovery but shows sustained expression in stricture formation. **A–C:** Representative image of dual-label immunocytochemistry showing colocalization of staining for PDGFRβ (**A**) with smooth muscle cells identified by staining for α-smooth muscle actin (SMA) (**B**) within the smooth muscle layer of the strictured region at day 16 (D16) post-trinitrobenzenesulfonic acid (TNBS) instillation. The merged image (**C**) shows PDGFR-β in red and SMA in green, with nuclei in blue (nuclear fluorochrome H333258). **Arrows** indicate overlap of staining of smooth muscle cell margins with PDGFR-β. **D:** Typical image showing general absence of PDGFR-β (red) expression in the D16 recovery colon, without overlap with α-smooth muscle actin (SMA) (green). **Arrows** indicate PDGFR-β expression by pericytes of a small blood vessel. **E:** Quantification of coexpression of PDGFRβ and SMA during the time course of TNBS colitis. Extensive colocalization at day 6 post-TNBS, which was maintained with subsequent stricture formation. No colocalization is seen in recovery animals. **P* < 0.05 versus control. Scale bars: 15 μm (**A** and **B**); 15 μm (**C**); 30 μm (**D**).

Although PCNA labeling of smooth muscle was undetectable in the control colon (Figure 6A), this was $0.05 \pm 0.03\%$ on day 6 ($n = 4$) and was subsequently undetectable in ISMC in D16 recovery animals, or in D35 or D90 nonstrictured animals. However, PCNA labeling of ISMC was prominent in the D16 stricture tissue, as well as in D35 and D90 strictures, and ranged from 0.4% to 1.2% (Figure 6B). Therefore, the continued inflammation in the smooth muscle layers at these times was also associated with ongoing smooth muscle proliferation.

The wave of cell division during the early stage of TNBS is a transient event, but the continued proliferation in the strictured region might lead to an ever-expanding smooth muscle mass if unchecked. Because cell loss might counteract the ongoing growth process, evidence for increased apoptosis was examined using dual-label immunocytochemistry for cleaved caspase-3 (CC3; a key marker of apoptosis³¹) and smooth muscle actin. This was absent from smooth muscle in sections of control colon, although anti-CC3 staining was typically present in epithelial cells at the mucosal verge (Figure 6C) (a positive control). A low, but consistent, level of anti-CC3 staining of smooth muscle was detectable by D6 of TNBS, but this virtually disappeared in D16 recovery and D35 nonstrictured tissues, whereas the developing and established strictures showed a relatively high presence of anti-CC3 labeling (Figure 6, C and D). Although variable among animals, the average incidence of apoptosis was typically higher than that seen during initial inflammation, and its continued presence could represent a major balancing force to the outcome of ongoing smooth muscle proliferation in the stricturing process.

PDGFRβ Is Expressed in Smooth Muscle Cells in the Strictured Region

The proliferation of ISMC in the inflamed intestine, and in primary cultures *in vitro*, is initiated by the expression of PDGFR-β in response to proinflammatory cytokines.^{11,12} Continued expression could also be a feature of proliferating smooth muscle cells in the developing and established intestinal stricture. Dual-label immunocytochemistry used antibodies to PDGFR-β combined with anti-smooth muscle actin to ensure smooth muscle-specific expression. Although no positive staining was seen in controls, this increased to approximately 15% of ISMC on D6 (Figure 7E), in line with earlier work,¹² and then declined to a near-undetectable levels in D16 recovery animals and D35 nonstrictured animals (Figure 7D). However, PDGFR-β staining remained characteristically high in the developing stricture on D16 (Figure 7, A–C), as well as in established strictures on D35 and D90 (Figure 7E). PECAM staining of adjacent sections was used to verify that PDGFR-β/SMA-positive cells were not vascular smooth muscle, as used previously.¹²

HIF1a Expression Is an Adaptive Strategy for ISMC Growth in the Stricturing Process

In the inflamed intestine, both smooth muscle proliferation and the metabolic challenges of inflammation may cause local ischemia, in part through challenges to the microvascular supply of oxygen. Elsewhere, ischemia typically causes stabilization of HIF-1α, causing transcription of

diverse genes that affect cell survival and metabolism in hypoxia.³² Although hypoxia in the mucosa has been described in TNBS colitis,³³ very little is known about its occurrence in the deeper intestinal layers (Joanne Kearon, MSc thesis, Queen's University, 2014), and so this was examined for involvement in stricture formation.

In tissues removed from animals at D6 of TNBS-induced inflammation, immunocytochemistry for HIF-1 α showed prominent labeling of CSM nuclei, with no staining in control sections (Figure 8, A and B), evidence for hypoxia in the smooth muscle layers. Notably, HIF-1 α staining was even further increased in the ISMC of forming or established strictures, whereas it decreased to near-absent levels in nonstrictured recovery tissues (ie, days 16 and 35 post-colitis) (Figure 8, C–E). This implies that the normally transient activation of HIF-1 α can become associated with the sustained inflammatory response in stricture formation.

Because HIF-1 α promotes cell survival, tissue culture was used to look for functional evidence that HIF-1 α was associated with survival and growth of ISMC in ischemia. Cultured CSM cell lines were derived from adult rat colon (1 line per animal) and studied at both low- and high-passage numbers, because it was recently reported that phenotypic alterations occur in high-passage cell lines that resemble features of the intestinal stricture *in vivo*.¹³ Low-passage cell lines displayed low levels of cytoplasmic HIF-1 α expression, whereas high-passage cell lines showed consistently strong nuclear staining, evidence for chronic HIF-1 α stabilization despite the normoxic culture conditions (Figure 8, F–I).

The functional consequences of HIF-1 α stabilization were then tested in several ways. First, the effect of inhibition of HIF-1 α signaling by the inhibitor chetomin on ISMC proliferation was tested in high-passage lines. This was highly sensitive to pretreatment with chetomin, with ≤ 10 nmol/L reducing the response to 5% serum to baseline (Figure 8J). As expected, similar testing of the HIF-1 α inducer cobalt chloride had no effect (Figure 8K). This suggested that ongoing HIF-1 α stabilization and nuclear signaling can promote ISMC proliferation in normoxic conditions. Two model systems were then used to look for functional outcomes that are representative of hypoxia or metabolic challenge. First, challenge with the metabolic inhibitor dinitrophenol was used to show a concentration-dependent decrease in ISMC survival in low-passage cell lines, but with no effect on high-passage lines (Figure 8L), implying a HIF-1 α -mediated effect. Next, experimental hypoxia (1% O₂ for 24 hours) showed that high-passage cell lines with stable high levels of HIF-1 α could continue to survive and proliferate, in contrast to low-passage cell lines where the cell number declined sharply (Figure 8M). Therefore, the onset of constitutive HIF-1 α stabilization strongly supports ISMC survival and growth in ischemic conditions and may represent a key adaptive strategy in stricture development.

The advantage of HIF-1 α to cell growth in the poorly vascularized tumor is well recognized, but both HIF-1 α and

TGF β participate in regulation of the altered extracellular matrix of the tumor microenvironment,^{34,35} which might resemble outcomes described here. In general, TGF β is a critical factor in the transition of the inflammatory response from acute damage to tissue healing, working in part by inducing the appearance of the alternatively activated or M2 macrophage phenotype and by directly influencing extracellular matrix expression.³⁶ Because the developing stricture displayed both an expanded smooth muscle cell population and increased collagen expression despite the presence of an M2 macrophage phenotype, as well as HIF-1 α up-regulation, the possibility of a dysregulated response to TGF β as a driving force in the forming stricture was tested.

The growth responses of ISMC were first compared, finding a nearly twofold increase in response of high- versus low-passage lines to either serum or the major mesenchymal growth factor PDGF-BB¹² (Figure 9A). This was also apparent in limited experiments where early-passage cell lines were derived from single animals with strictures that allowed comparison of the growth response to PDGF of cells from the stricture versus the adjacent normal tissue (Figure 9B).

TGF β was highly effective in suppressing the growth response to PDGF of low-passage ISMC, by either pretreatment or concurrent application, whereas high-passage ISMC were completely nonresponsive (Figure 9C). Nonetheless, Western blotting showed that TGF β induced rapid, transient up-regulation of pSMAD2/3 in low- and high-passage ISMC, thus verifying the transduction presence of signal in both cases (Figure 9D). Overall, this is evidence that TGF β may exert only partial influence on smooth muscle phenotype and growth in the stricture due to defective subcellular signaling.

In conclusion, multiple lines of evidence suggest that persistent inflammation creates and maintains the intestinal stricture, which is characterized by the presence of an altered smooth muscle phenotype that can survive and proliferate in an ischemic inflammatory environment.

Discussion

The evolution of intestinal strictures in CD is an unclear process, occurring in the context of a disease that is itself sporadic. For example, it is not well predicted by genetic risk factors, such as mutations in *NOD2* or *ATG16L*, which show clear association but are neither sufficient nor necessary for disease occurrence.^{1,37,38} Similarly, there is no defined precipitating factor for the frequent outcome of stricture formation and intestinal obstruction, where the pathogenesis involves both increased wall thickness^{16,39} and altered tissue compliance.^{40,41} This arises from a common process of smooth muscle cell proliferation and a modulated cellular phenotype,^{13,42} recently gaining acknowledgment for its role in causing wall thickening and obstructive symptoms.^{2,16,43} This expanded cellular compartment is a potential target for

intervention, but more significantly, it is a site for epigenetic change as a consequence of proliferation, which is shown to be at least experimentally reversible.¹³

Animal models have given valuable insight into CD, involving defined challenges to gut integrity, such as genetic or cellular approaches, that cause inflammation but typically do not recapitulate stricturing as a locally severe and regionally limited event. However, among the chemical

models of intestinal inflammation, the TNBS model of colitis in the Sprague-Dawley rat is unique in causing sporadic stricture formation, as reported earlier.¹⁸ Here, this research has identified the elements of developing strictures in the late resolution phase of inflammation, with the key observations of unresolved transmural inflammation restricted to a region with ongoing mucosal inflammation and a characteristic M2 macrophage presence in the smooth muscle layers.

Stricture formation *in vivo* showed unique features that appeared during the initial inflammation but then persisted over time in some animals. These included an ongoing expression of PDGF-R β that correlated with smooth muscle proliferation, along with decreased expression of smooth muscle marker proteins. A constitutively increased appearance of HIF-1 α may further promote tissue expansion under ischemic conditions. The developing and established strictures showed markedly increased expression of collagen III protein, similar to Crohn strictures, although this remained a small proportion of tissue area, again similar to the human equivalent.^{39,43} This seems due to the progressive modulation of smooth muscle phenotype that generates an altered nature and amount of collagen expression. Overall, the early appearance of key cellular features of stricture formation indicates possible causal processes for examination in human tissues.

The sustained proliferation of ISMC in stricture development suggests that epigenetic modulation of gene expression contributes to the altered phenotype *in vivo*, as described in rat ISMC and human CD strictures.¹³ The current study identified reduced expression of both SM22 α and smoothelin in D16 strictured tissue and a further decrease by D35 post-TNBS, which parallels the results of growth *in vitro* of both rat and human ISMC shown earlier. Significantly, human ISMC isolated from Crohn strictures already showed similar changes, implying that a parallel process occurred *in vivo* in CD.¹³

The decreased expression of the smooth muscle markers (ie, SM22 α , smoothelin, α SMA) may be evidence for an altered cellular phenotype with consequences that extend beyond impaired contractility. For example, SM22 α induces F-actin bundle formation and normally maintains the differentiated phenotype of vascular smooth muscle cells.⁴⁴ Further, sustained proliferation of rat ISMC *in vitro* caused the loss of expression of the key neurotrophin GDNF,⁴⁵ which could be experimentally reversed by inhibition of DNA methyltransferase 1 or histone deacetylase.¹³ This is interpreted to explain the characteristic loss of innervation reported earlier in this rat stricture model.¹⁸

Much of the observed outcome of an altered smooth muscle phenotype may be a direct outcome of PDGF-BB

signaling, occurring here and in earlier studies,¹² and also described in Crohn strictures.⁴³ PDGF-BB is the most potent mitogen for intestinal and other smooth muscle cell types, where it also exerts wide-ranging effects on gene expression, typically promoting protein synthesis and inhibiting expression of contractile elements.^{46,47} PDGF-BB is implicated in tissue remodeling in disease conditions affecting the airway, vasculature and intestine among others, and inhibition of signaling has benefit in limiting or preventing disease severity.^{48,49} However, the role of PDGF has not yet been addressed in CD, although supported by preliminary human and animal model work.^{50,51}

The novel finding of stable expression of HIF-1 α in the smooth muscle of rat strictures may also be an outcome of PDGF-induced signaling, because this was among the top transcription factors induced by PDGF-BB in rat vascular smooth muscle,⁵² although not yet specifically studied in rat ISMCs. In addition, PDGF induces HIF-1 α expression in airway smooth muscle that is associated with a switch to glycolysis (the Warburg effect).^{53,54} Its characteristic presence in high-passage cultured ISMCs was found to be associated with continued survival and proliferation under ischemic challenge, suggestive of a glycolytic phenotype,^{55,56} and further, that the sustained HIF-1 α presence was required for the hyperproliferative growth response to PDGF of these cells. The uniform acquisition of this phenotype by all high-passage cell lines, even though independently created from separate adult rats, shows that the growth-dependent modulation of phenotype converges on this outcome. This further supports the probability that epigenetic change *in vivo* is responsible for the emergence of a HIF-1 α -dependent, PDGF-driven phenotype of smooth muscle in the intestinal stricture, adding to the information derived earlier in human smooth muscle in Crohn strictures.¹³

In the current study, the sustained expression of PDGF-R β in CSM in a hypoxic inflamed environment may explain an increased responsiveness to environmental stimuli containing growth factors such as PDGF-BB. However, this is simplistic, because inflammation also causes exactly these changes in the acute response to TNBS-induced colitis,¹¹ normally followed

Figure 8 Hypoxia inducible factor 1 subunit alpha (HIF-1 α) is transiently mobilized to the smooth muscle nucleus during colitis, but persists in smooth muscle in stricture formation, and is associated with increased cell survival and proliferation in ischemia *in vitro*. **A–D:** Immunohistochemistry for HIF-1 α (red) and smooth muscle actin (SMA) (green) in the circular smooth muscle of the colon, showing the absence of HIF-1 α in control (**A**), and its appearance in colitis on day 6 (D6). Although absent from D16 and D35 recovery animals, HIF-1 α is abundant in smooth muscle cells in D16 and D35 strictures (**B–D**; arrows). **E:** Nuclear mobilization of HIF-1 α in intestinal smooth muscle cells (ISMC) in trinitrobenzenesulfonic acid (TNBS)-induced colitis. High levels in colitis on D6 drop sharply in D16 and D35 recovery animals (note discontinuous y axis) but remain high in the strictured regions. **F–I:** Images showing absence of nuclear HIF-1 α in early passage adult colonic circular smooth muscle cell (P3 ISMC) lines (**F** and arrow in **G**), in contrast to stable nuclear translocation of HIF-1 α in high-passage ISMC cell lines (P13) (**H** and arrow in **I**) that mimic the altered phenotype seen in strictures *in vivo*. **J** and **K:** Pretreatment of high-passage ISMC lines with chetomin, an inhibitor of HIF-1 α signaling, blocks subsequent proliferation in response to fetal calf serum (FCS) (5%) at low nanomolar concentrations (**J**, note semilog axis), whereas similar pretreatment with the HIF-1 α inducer cobalt chloride (CoCl₂) has no effect over the range of 10 to 200 μ mol/L (**K**). **L:** High-passage cell lines of ISMC (red) survive and grow during exposure to the metabolic inhibitor dinitrophenol (DNP; 50 to 750 μ mol/L). By contrast, low-passage cell lines (blue) show increasing cytotoxicity with increased DNP levels. **M:** Differential response to hypoxic challenge in high- versus low-passage ISMC lines. High-passage cell lines expressing HIF-1 α (red) grow readily during exposure to hypoxic conditions for 48 hours, whereas low-passage ISMC cell lines (blue) decrease in cell number. Each cell line was derived from a separate adult male rat. Data are expressed as means $\pm$ SEM (**K** and **L**). $n = 3$ ISMC cell lines (**J** and **K**); $n = 3$ to 4 cell lines (**L**); $n = 3$ to 5 replicates (**M**). * $P < 0.05$ versus time-matched cohorts; [†] $P < 0.05$ versus high passage at the same dosage. Scale bars: 20 μ m (**A–D**); 12 μ m (**F** and **H**); 4 μ m (**G** and **I**).

by resolution without stricture formation in the majority of animals. This is better explained by the dual findings of TGFβ-mediated suppression of growth in low-passage ISMC, that crucially is lost in the high-passage phenotype. The authors propose that TGFβ in the resolution phase of colitis normally suppresses smooth muscle proliferation and promotes the restoration of the contractile phenotype, but that an unpredictable loss of sensitivity to TGFβ in a subpopulation of dividing cells goes on to cause stricture formation. Again, this points to epigenetic change as the culprit in stricture formation, aided by unclear contributions from the environment and microbiome in both the animal model and in CD.

The persistent inflammation in the developing and mature stricture showed several defining characteristics that may reflect the causal factors for stricture formation. For example, the marked infiltration of eosinophils in the strictured colon at D16 post-TNBS has been documented in Crohn strictures,⁵⁷ and eosinophil cell sonicates have been shown to increase proliferation and collagen expression in myofibroblasts from Crohn strictures,⁵⁸ suggesting that eosinophil-derived factors may participate in smooth muscle growth. Also, a large T-cell presence was found in the developing and stable strictures, reaching levels higher than those on day 6 of active inflammation and notably absent from nonstrictured cohort animals. Within the spectrum of T-helper cell–derived fates, this is expected to reflect a TH-1 dominant profile as described for TNBS-induced colitis⁵⁹ and viewed as characteristic for CD.⁶⁰ The possibility of tissue-resident, memory phenotype T cells raises the further question of the unknown nature of antigen-specific stimuli that may promote relapsing disease.

The presence of abundant CD68-positive macrophages among the smooth muscle cells of the stricture (again in strong contrast to their near-absence in nonstrictured cohorts) suggested an active inflammatory response likely to display an M1 phenotype. However, in pursuing this, no supportive evidence was found, although a consistent pattern of iNOS

Figure 9 High-passage intestinal smooth muscle cells (ISMC) are hyperproliferative to growth factors but nonresponsive to growth suppression by transforming growth factor beta (TGFβ), compared with low-passage cell lines. **A:** Cell proliferation assays showing that high-passage ISMC lines are substantially more responsive to serum [fetal calf serum (FCS), left] or to platelet-derived growth factor (PDGF) (50 ng/mL, right) than low passage cells lines in parallel assays. **B:** Within-animal comparison of growth responses. Early-passage cell lines (P2) from the strictured region versus adjacent nonaffected region in matched animals are hyperresponsive to PDGF over a wide range of concentrations. **C:** Effect of pretreatment or concurrent treatment with TGFβ (50 ng/mL) of low- versus high-passage ISMC cell lines exposed to PDGF (50 ng/mL). Low-passage ISMC shows significant inhibition of growth response with TGFβ pretreatment or combined treatment, whereas high-passage cell lines are nonresponsive. Data normalized to the PDGF response in each case (left). **D:** Western blots showing up-regulation of the signaling intermediate phosphorylated SMAD family member pSMAD2/3 by 30 minutes post-TGFβ in both low- and high-passage cell lines. Note the strong signal in high-passage cell lines and loading control of β-actin. *n* = 5 cell lines per condition (**A**) and cell lines in triplicate assay (**C**); *n* = 2 cell lines (**B**); *n* = 3 cell lines per condition (**D**). **P* < 0.05 versus high passage.

expression that did not overlap with CD68 appeared in immune cells that were tightly localized to the subepithelial region. Unexpectedly, this study found an M2 phenotype of macrophages in the underlying smooth muscle.

In some regards, it is challenging to clearly distinguish this from the contribution of normally tissue-resident macrophages, because several markers of the M2 phenotype overlap with this population; indeed, CD163 and CD206 are markers for these.²² However, immunoreactivity for arginase, the key M2 marker, was uniquely detected in the developing and established stricture, and is a hallmark for the M2 macrophage. Conceivably, the marked up-regulation of the tissue resident and professional M2 phenotypes that was identified might derive from skewing of differentiation of blood monocyte-derived precursors in the developing stricture, but could also be a causal event for ongoing tissue remodeling—resolution of this interaction among the underlying diffusible or matrix-related factors remains a future goal. In either case, the M2 phenotype is found in fibrotic areas of tissue samples from CD patients,⁶¹ and, among other actions, can activate cells to produce extracellular matrix,⁶² with arginase activity supporting collagen biosynthesis via proline production.⁶³ In the current animal model, M2-mediated events in the deeper regions may oppose the on-going inflammatory processes in the mucosa.

In summary, stricture formation arises in a Crohn-like model of colitis in the rat, and study of an earlier time point has identified characteristic cellular phenomena, including a persistent M2-like phenotype of macrophages and on-going ISMC proliferation. An unresolved mucosal inflammation is associated with thickening of the underlying smooth muscle layer, where proliferation drives epigenetic modifications to structural and functional aspects of ISMC that comprise the central pathology. This includes the ongoing expression of HIF-1 α and the failure of the anti-proliferative role of TGF β , potential markers for use in predicting the likelihood of stricture recurrence in CD and identifying cellular targets for more specific forms of anti-inflammatory therapy.

References

1. Torres J, Mehandru S, Colombel JF, Peyrin-Biroulet L: Crohn's disease. *Lancet* 2017, 389:1741–1755
2. Li J, Mao R, Kurada S, Wang J, Lin S, Chandra J, Rieder F: Pathogenesis of fibrostenosing Crohn's disease. *Transl Res* 2019, 209: 39–54
3. Ma C, Jairath V, Click B, Hirota SA, Lu C, Parker CE, Rieder F: Stenosis Therapy and Anti-Fibrotic Research (STAR) Consortium: Targeting anti-fibrotic pathways in Crohn's disease - the final frontier? *Best Pract Res Clin Gastroenterol* 2019, 38-39:101603
4. de Bruyn JR, Meijer SL, Wildenberg ME, Bemelman WA, van den Brink GR, D'Haens GR: Development of fibrosis in acute and longstanding ulcerative colitis. *J Crohns Colitis* 2015, 9:966–972
5. Burke JP, Mulsow JJ, O'Keane C, Docherty NG, Watson RW, O'Connell PR: Fibrogenesis in Crohn's disease. *Am J Gastroenterol* 2007, 102:439–448
6. Dietz DW, Laureti S, Strong SA, Hull TL, Church J, Remzi FH, Lavery IC, Fazio VW: Safety and longterm efficacy of strictureplasty

- in 314 patients with obstructing small bowel Crohn's disease. *J Am Coll Surg* 2001, 192:330–337
7. Antoniou E, Margonis GA, Angelou A, Pikouli A, Argiri P, Karavokyros I, Papalois A, Pikoulis E: The TNBS-induced colitis animal model: an overview. *Ann Med Surg (Lond)* 2016, 11:9–15
8. Blennerhassett MG, Lourens SR, Parlow LRG, Ghasemlou N, Winterborn AN: Analgesia and mouse strain influence neuromuscular plasticity in inflamed intestine. *Neurogastroenterol Motil* 2017, 29:1–12
9. Lourens S, Wells RW, Blennerhassett MG: Differential responses of intrinsic and extrinsic innervation of smooth muscle cells in rat colitis. *Exp Neurol* 2005, 195:497–507
10. Nair DG, Han TY, Lourens S, Blennerhassett MG: Proliferation modulates intestinal smooth muscle phenotype in vitro and in colitis in vivo. *Am J Physiol Gastrointest Liver Physiol* 2011, 300: G903–G913
11. Nair DG, Miller KG, Lourens SR, Blennerhassett MG: Inflammatory cytokines promote growth of intestinal smooth muscle cells by induced expression of PDGF-Rbeta. *J Cell Mol Med* 2014, 18:444–454
12. Stanzel RDP, Lourens S, Nair DG, Blennerhassett MG: Mitogenic factors promoting intestinal smooth muscle cell proliferation. *Am J Physiol Cell Physiol* 2010, 299:C805–C817
13. Bonafiglia QA, Lourens SR, Hurlbut DJ, Blennerhassett MG: Epigenetic modification of intestinal smooth muscle cell phenotype during proliferation. *Am J Physiol Cell Physiol* 2018, 315: C722–C733
14. Van Assche G, Geboes K, Rutgeerts P: Medical therapy for Crohn's disease strictures. *Inflamm Bowel Dis* 2004, 10:55–60
15. Flynn RS, Murthy KS, Grider JR, Kellum JM, Kuemmerle JF: Endogenous IGF-I and alphaVbeta3 integrin ligands regulate increased smooth muscle hyperplasia in stricturing Crohn's disease. *Gastroenterology* 2010, 138:285–293
16. Chen W, Lu C, Hirota C, Iacucci M, Ghosh S, Gui X: Smooth muscle hyperplasia/hypertrophy is the most prominent histological change in Crohn's fibrostenosing bowel strictures: a semiquantitative analysis by using a novel histological grading scheme. *J Crohns Colitis* 2017, 11:92–104
17. Suekane T, Ikura Y, Watanabe K, Arimoto J, Iwasa Y, Sugama Y, Kayo S, Sugioka K, Naruko T, Maeda K, Hirakawa K, Arakawa T, Ueda M: Phenotypic change and accumulation of smooth muscle cells in strictures in Crohn's disease: relevance to local angiotensin II system. *J Gastroenterol* 2010, 45:821–830
18. Marlow SL, Blennerhassett MG: Deficient innervation characterizes intestinal strictures in a rat model of colitis. *Exp Mol Pathol* 2006, 80: 54–66
19. Roszer T: Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators Inflamm* 2015, 2015:816460
20. Shiraishi M, Shintani Y, Shintani Y, Ishida H, Saba R, Yamaguchi A, Adachi H, Yashiro K, Suzuki K: Alternatively activated macrophages determine repair of the infarcted adult murine heart. *J Clin Invest* 2016, 126:2151–2166
21. Lau SK, Chu PG, Weiss LM: CD163: a specific marker of macrophages in paraffin-embedded tissue samples. *Am J Clin Pathol* 2004, 122:794–801
22. Viola MF, Boeckxstaens G: Intestinal resident macrophages: multitaskers of the gut. *Neurogastroenterol Motil* 2020, 32:e13843
23. Lawrence T, Natoli G: Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol* 2011, 11:750–761
24. Li L, Miano JM, Cserjesi P, Olson EN: SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ Res* 1996, 78:188–195
25. Gomez D, Owens GK: Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res* 2012, 95:156–164
26. Graham MF, Diegelmann RF, Elson CO, Lindblad WJ, Gotschalk N, Gay S, Gay R: Collagen content and types in the intestinal strictures of Crohn's disease. *Gastroenterology* 1988, 94:257–265

27. Mourelle M, Salas A, Guarner F, Crespo E, Garcia-Lafuente A, Malagelada JR: Stimulation of transforming growth factor beta1 by enteric bacteria in the pathogenesis of rat intestinal fibrosis. *Gastroenterology* 1998, 114:519–526
28. Graham MF, Drucker DE, Diegelmann RF, Elson CO: Collagen synthesis by human intestinal smooth muscle cells in culture. *Gastroenterology* 1987, 92:400–405
29. Garcia P, Schmiedlin-Ren P, Mathias JS, Tang H, Christman GM, Zimmermann EM: Resveratrol causes cell cycle arrest, decreased collagen synthesis, and apoptosis in rat intestinal smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 2012, 302:G326–G335
30. Beresford MJ, Wilson GD, Makris A: Measuring proliferation in breast cancer: practicalities and applications. *Breast Cancer Res* 2006, 8:216
31. Nagata S: Apoptosis and clearance of apoptotic cells. *Annu Rev Immunol* 2018, 36:489–517
32. Thomas LW, Ashcroft M: Exploring the molecular interface between hypoxia-inducible factor signalling and mitochondria. *Cell Mol Life Sci* 2019, 76:1759–1777
33. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH: Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J Clin Invest* 2004, 114:1098–1106
34. Ando A, Hashimoto N, Sakamoto K, Omote N, Miyazaki S, Nakahara Y, Imaizumi K, Kawabe T, Hasegawa Y: Repressive role of stabilized hypoxia inducible factor 1alpha expression on transforming growth factor beta-induced extracellular matrix production in lung cancer cells. *Cancer Sci* 2019, 110:1959–1973
35. Gjaltema RAF, de Rond S, Rots MG, Bank RA: Procollagen lysyl hydroxylase 2 expression is regulated by an alternative downstream transforming growth factor beta-1 activation mechanism. *J Biol Chem* 2015, 290:28465–28476
36. Sanjabi S, Oh SA, Li MO: Regulation of the immune response by TGF-beta: from conception to autoimmunity and infection. *Cold Spring Harb Perspect Biol* 2017, 9:a022236
37. Torres J, Colombel JF: Genetics and phenotypes in inflammatory bowel disease. *Lancet* 2016, 387:98–100
38. Uniken Venema WT, Voskuil MD, Dijkstra G, Weersma RK, Festen EA: The genetic background of inflammatory bowel disease: from correlation to causality. *J Pathol* 2017, 241:146–158
39. Zhang X, Ko HM, Torres J, Panchal HJ, Cai Z, Wagner M, Sands BE, Colombel JF, Cho J, Taouli B, Harpaz N: Luminally polarized mural and vascular remodeling in ileal strictures of Crohn's disease. *Hum Pathol* 2018, 79:42–49
40. Kim K, Johnson LA, Jia C, Joyce JC, Rangwalla S, Higgins PD, Rubin JM: Noninvasive ultrasound elasticity imaging (UEI) of Crohn's disease: animal model. *Ultrasound Med Biol* 2008, 34:902–912
41. Stidham RW, Higgins PD: Imaging of intestinal fibrosis: current challenges and future methods. *United Eur Gastroenterol J* 2016, 4: 515–522
42. Li C, Vu K, Hazelgrove K, Kuemmerle JF: Increased IGF-IEC expression and mechano-growth factor production in intestinal muscle of fibrostenotic Crohn's disease and smooth muscle hypertrophy. *Am J Physiol Gastrointest Liver Physiol* 2015, 309: G888–G899
43. Severi C, Sferri R, Scirocco A, Vetusch A, Pallotta N, Pronio A, Caronna R, Di Rocco G, Gaudio E, Corazziari E, Onori P: Contribution of intestinal smooth muscle to Crohn's disease fibrogenesis. *Eur J Histochem* 2014, 58:2457
44. Han M, Dong L-H, Zheng B, Shi J-H, Wen J-K, Cheng Y: Smooth muscle 22 alpha maintains the differentiated phenotype of vascular smooth muscle cells by inducing filamentous actin bundling. *Life Sci* 2009, 84:394–401
45. Han TY, Lourenssen S, Miller KG, Blennerhassett MG: Intestinal smooth muscle phenotype determines enteric neuronal survival via GDNF expression. *Neuroscience* 2015, 290:357–368
46. Raines EW: PDGF and cardiovascular disease. *Cytokine Growth Factor Rev* 2004, 15:237–254
47. Kaplan-Albuquerque N, Bogaert YE, Van Putten V, Weiser-Evans MC, Nemenoff RA: Patterns of gene expression differentially regulated by platelet-derived growth factor and hypertrophic stimuli in vascular smooth muscle cells: markers for phenotypic modulation and response to injury. *J Biol Chem* 2005, 280:19966–19976
48. Ishii Y, Hamashima T, Yamamoto S, Sasahara M: Pathogenetic significance and possibility as a therapeutic target of platelet derived growth factor. *Pathol Int* 2017, 67:235–246
49. Papadopoulos N, Lennartsson J: The PDGF/PDGFR pathway as a drug target. *Mol Aspects Med* 2018, 62:75–88
50. Langer V, Vivi E, Regensburger D, Winkler TH, Waldner MJ, Rath T, Schmid B, Skottke L, Lee S, Jeon NL, Wohlfahrt T, Kramer V, Tripal P, Schumann M, Kersting S, Handtrack C, Geppert CI, Suchowski K, Adams RH, Becker C, Ramming A, Naschberger E, Britzen-Laurent N, Sturz M: IFN-gamma drives inflammatory bowel disease pathogenesis through VE-cadherin-directed vascular barrier disruption. *J Clin Invest* 2019, 129:4691–4707
51. Magro F, Costa C: Long-standing remission of Crohn's disease under imatinib therapy in a patient with Crohn's disease. *Inflamm Bowel Dis* 2006, 12:1087–1089
52. Chen J, Cui X, Qian Z, Li Y, Kang K, Qu J, Li L, Gou D: Multi-omics analysis reveals regulators of the response to PDGF-BB treatment in pulmonary artery smooth muscle cells. *BMC Genomics* 2016, 17:781
53. Li B, Zhu Y, Sun Q, Yu C, Chen L, Tian Y, Yan J: Reversal of the Warburg effect with DCA in PDGF-treated human PASMC is potentiated by pyruvate dehydrogenase kinase-1 inhibition mediated through blocking Akt/GSK-3beta signalling. *Int J Mol Med* 2018, 42: 1391–1400
54. Xiao Y, Peng H, Hong C, Chen Z, Deng X, Wang A, Yang F, Yang L, Chen C, Qin X: PDGF promotes the Warburg effect in pulmonary arterial smooth muscle cells via activation of the PI3K/AKT/mTOR/HIF-1alpha signaling pathway. *Cell Physiol Biochem* 2017, 42:1603–1613
55. Dai J, Zhou Q, Chen J, Rexius-Hall ML, Rehman J, Zhou G: Alpha-enolase regulates the malignant phenotype of pulmonary artery smooth muscle cells via the AMPK-Akt pathway. *Nat Commun* 2018, 9:3850
56. Thenappan T, Ormiston ML, Ryan JJ, Archer SL: Pulmonary arterial hypertension: pathogenesis and clinical management. *BMJ* 2018, 360:j5492
57. Masterson JC, Capocelli KE, Hosford L, Biette K, McNamee EN, de Zoeten EF, Harris R, Fernando SD, Jedlicka P, Protheroe C, Lee JJ, Furuta GT: Eosinophils and IL-33 perpetuate chronic inflammation and fibrosis in a pediatric population with stricturing Crohn's ileitis. *Inflamm Bowel Dis* 2015, 21:2429–2440
58. Xu X, Rivkind A, Pikarsky A, Pappo O, Bischoff SC, Levi-Schaffer F: Mast cells and eosinophils have a potential profibrogenic role in Crohn disease. *Scand J Gastroenterol* 2004, 39: 440–447
59. Galitovskiy V, Qian J, Chernyavsky AI, Marchenko S, Gindi V, Edwards RA, Grando SA: Cytokine-induced alterations of alpha7 nicotinic receptor in colonic CD4 T cells mediate dichotomous response to nicotine in murine models of Th1/Th17- versus Th2-mediated colitis. *J Immunol* 2011, 187:2677–2687
60. Brand S: Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut* 2009, 58:1152–1167
61. Scharl M, Huber N, Lang S, Furst A, Jehle E, Rogler G: Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. *Clin Transl Med* 2015, 4:1
62. Manresa MC, Godson C, Taylor CT: Hypoxia-sensitive pathways in inflammation-driven fibrosis. *Am J Physiol Regul Integr Comp Physiol* 2014, 307:R1369–R1380
63. Durante W, Johnson FK, Johnson RA: Arginase: a critical regulator of nitric oxide synthesis and vascular function. *Clin Exp Pharmacol Physiol* 2007, 34:906–911