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RESEARCH PAPER

PDGF and TGF-β promote tenascin-C expression in subepithelial myofibroblasts and contribute to intestinal mucosal protection in mice

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BACKGROUND AND PURPOSE

Tenascin-C (TnC) is a multi-domain extracellular matrix glycoprotein that is expressed at a high level during embryogenesis but is almost absent during normal postnatal life. This multi-domain complex molecule is reported to associate with both pro-inflammatory and anti-inflammatory signalling cascades. In this study, we examined how TnC modulated intestinal inflammation.

EXPERIMENTAL APPROACH

TnC pathophysiology was evaluated in cultures of rat intestinal subepithelial myofibroblasts (ISEMF) and intestinal epithelial cells. Wild-type and TnC(–/–) mice were treated with dextran sodium sulfate (DSS) to induce colitis.

KEY RESULTS

DSS-induced colitis in mice markedly increased TnC in the damaged mucosal areas and up-regulated mRNA for TnC, pro-inflammatory cytokines and growth factors (PDGF-B and TGF-β1). In addition, 2,4,6-trinitrobenzene sulfonic acid-induced colitis and SAMP1/Yit mice, a model of spontaneous Crohn's disease, also exhibited increased mucosal TnC in colon and ilea respectively. PDGF receptor-α (PDGFRα) positive ISEMF were the primary TnC-producing cells in colon tissues. Accordingly, ISEMF collected from the rat colon constitutively expressed both TnC and PDGFRα. PDGF-BB and TGF-β1 up-regulated both TnC mRNA and protein levels in ISEMF. Knock-down of TnC gene increased susceptibility to DSS-induced colitis, compared with TnC(+/+) littermates. TnC(–/–) mice showed marked abrasion of intestinal mucosal barrier and increased inflammatory scores. Moreover, TnC accelerated both trans-well migration and wound healing in epithelial cells.

CONCLUSIONS AND IMPLICATIONS

The pharmacological profiles of PDGF-BB and TGF-β in colitis tissues and ISEMF suggest that increased TnC production during inflammation contributed to epithelial cell migration, remodelling and protection of intestinal barriers.

Abbreviations

β-gal, β-galactosidase; DSS, dextran sodium sulfate; IEC, intestinal epithelial cell; ISEMF, intestinal subepithelial myofibroblasts; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TnC, tenascin-C

Introduction

Tenascin-C (TnC) is an extracellular matrix protein that is expressed at a high level during embryogenesis but its expression is almost absent during normal post-natal life, with the exception of basal expression in various tissues. TnC expression re-appears under pathological conditions such as tissue injury, wound healing, vascular disease, tumorigenesis and metastasis (Jones and Jones, 2000a). Recent evidence suggests that TnC expression is increased in ulcerative colitis and Crohn's disease (Riedl *et al.*, 2001; Salas *et al.*, 2003; Griga *et al.*, 2004; Brain *et al.*, 2009). Although multiple functions have been assigned to TnC based on clinical studies, it is difficult to determine the exact roles of TnC without extensive *in vivo* and *in vitro* studies to determine the specific signalling regulation of TnC. The deletion of the TnC gene (TnC knock-out) in mice exacerbates spinal cord injury (Chen *et al.*, 2010), glomerulonephritis (Nakao *et al.*, 1998) and atherosclerosis (Wang *et al.*, 2012). Therefore, the TnC gene might play either pathological or protective roles through different signalling pathways.

It is important to determine the role of TnC in gut inflammation and the type of cells that are responsible for the constitutive and pathological expression of TnC. During mucosal inflammation, a mixture of cells of different lineages including epithelial cells, lymphocytes, resident macrophages, subepithelial fibroblasts and fibroblast-like cells are involved in either pro-inflammatory or anti-inflammatory events. In the present study, we focused on intestinal subepithelial myofibroblasts (ISEMF) as a source of TnC in the mucosa because myofibroblasts are inflammatory and secrete not only pro-inflammatory cytokines, chemokines, growth factors, and lipid and gaseous inflammatory mediators but also many extracellular matrix proteins. Thus, myofibroblasts play an important role in organogenesis, oncogenesis, inflammation and fibrosis in most organs and tissues (Valentich *et al.*, 1997; Powell *et al.*, 1999; Salas *et al.*, 2003).

We found that in experimentally induced intestinal inflammation in mice, TnC was up-regulated in the inflamed and damaged mucosal areas. Furthermore, TnC gene deletion exacerbated the colitis induced by dextran sodium sulfate (DSS), which revealed a protective role of TnC in gastrointestinal inflammation. The primary TnC-producing cell were ISEMF, which were also positive for the PDGF receptor-α (PDGFRα; receptor nomenclature follows Alexander *et al.*, 2013). We established that PDGF-BB and TGF-β1 trigger TnC production in ISEMF during colonic inflammation which influenced epithelial cell migration, remodelling and protection of intestinal barriers.

Methods

Animals

All animal care and experimental procedures were in accordance with the institutional guidelines of the University of Tokyo (approval code: P07-138). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 136 animals were used in the experiments described here. Sprague–Dawley SD rats (5 weeks old, male) were purchased from Charles River Laboratories (Wilmington, MA, USA). These rats were acclimatized and used for isolation of ISEMF from colon tissues. TnC-null mice were generated in our laboratory (Saga *et al.*, 1992) by targeted allele disruption in which most of exon 2 of the TnC gene is deleted and the *Escherichia coli* β-galactosidase (β-gal) gene (*LacZ*) is inserted. Heterozygous TnC(+/–) mice were intercrossed to yield TnC(–/–) and TnC(+/+) littermates on a C57BL/6N or Balb/C genetic background. Eight-week-old mice that weighed 20–22 g were used in the experiments. SAMP1/Yit (Yakult Central Institute for Microbiological Research, Tokyo, Japan) mice, which provide a model of spontaneous Crohn's disease (Matsumoto *et al.*, 1998) and age-matched AKR mice (healthy controls; Charles River, Yokohama, Japan) used to study the re-appearance of TnC during gastrointestinal inflammation. SAMP1/Yit and AKR mice were older than 20 weeks when used in experiments.

Induction of colitis in mice and evaluation of disease activity index

Experimental colitis was induced in TnC(+/+) and TnC(–/–) littermates by providing DSS (MP Biomedicals Inc., Osaka, Japan) in drinking water at 1% (w/v) for C57BL/6N mice or 2% for Balb/C mice, *ad libitum* for 3–8 days (Islam *et al.*, 2008). Body weight, stool consistency and stool blood were recorded daily. Disease activity index was determined by combining scores of (i) body weight loss, (ii) stool consistency and (iii) stool blood, divided by 3. Each score was determined as follows: change in body weight (0: <1%; 1: 1–5%; 2: 5–10%; 3: 10–15%, 4: >15%), stool blood (0: negative, 1: +, 2: ++, 3: +++, 4: ++++) and stool consistency (0: normal, 2: soft, 4: diarrhoea). In addition, we used another model of colitis in C57BL/6N mice, induced by 2, 4, 6-trinitrobenzene sulfonic acid (TNBS; Tokyo Kasei Kogyo,Tokyo, Japan). TNBS (150 $mg \cdot kg^{-1}$ . . . . .) was administered intrarectally under anaesthesia and samples were taken 2 days after TNBS administration.

LacZ *and immune-electron microscopic staining*

Activity of β-gal was assessed in colon tissues. Representative samples were fixed in 2% paraformaldehyde containing 1% glutaraldehyde and 0.2% Triton-X100 (Roche Diagnostic GmbH, Mannheim, Germany), stained with X-gal (Gibco BRL) and counter-stained with eosin. TnC(+/+) mice showed negative staining with X-gal (data not shown). *LacZ*-positive sections were then treated with 0.03% hydrogen peroxide, blocked in 10% normal goat serum and incubated with anti-smooth muscle α-actin (anti-αSMA; 1:500) and anti-PDGFRα (1:200) antibody respectively. The peroxidase activity was visualized with 3,3′-diaminobenzidine staining. For immune-electron microscopic staining, formaldehyde-fixed tissue sections were then post-fixed in 1% osmium tetroxide, stained with uranyl acetate, dehydrated and embedded in Epon. Ultrathin sections were cut with an ultramicrotome and observed with a Hitachi H-7650 transmission electron microscope.

Immunofluorescent staining of colon tissue from mice

Representative specimens from the mid-colon were permeabilized with Triton X-100 (0.2%), blocked by 10% normal goat

serum and then incubated with the following primary antibodies: rat anti-TnC (1:500 dilution), rabbit anti-αSMA (1:1000 dilution), monoclonal mouse anti-human αSMA (1:1000 dilution), goat anti-PDGFRα (1:200 dilution) and rabbit anti β-gal (1:500 dilution) antibody respectively. After washing with PBS, the specimens were incubated with the appropriate secondary antibodies (1:1000 dilution) for 2 h and counter-stained with DAPI (Sigma Aldrich, Tokyo, Japan).

Isolation of ISEMF

ISEMF were isolated from rat colon mucosa (Ohama *et al.*, 2008). Mucosal sheets were treated with 1 mM EDTA in Ca^{2+}-free HBSS buffer three times at 37°C for 30 min to remove the epithelial layer. The mucosal sheet without epithelial cells was then cut into small pieces and cultured in DMEM containing 10% FBS and 1% antimycotic/antibiotic at 37°C in 5% CO_2/95% O_2. The cultured ISEMF were used for experiments after three to five passages.

Immunofluorescent staining of ISEMF

ISEMF were incubated with rabbit polyclonal α-hTnC (1:250) and mouse monoclonal anti-αSMA (1:500). All specimens were washed and incubated then with the appropriate secondary antibodies diluted 1:1000 respectively for 2 h at room temperature in a dark chamber. Nuclei were stained with DAPI. Images were obtained using an Eclipse E800 fluorescence microscope (Nikon, Tokyo, Japan).

Western blotting analysis for ISEMF

Conditioned medium and cell lysates were used for Western blot analysis (Andrews *et al.*, 2009). Cell lysates were prepared in extraction buffer containing 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, complete protease inhibitor (Roche) and Pefabloc SC (Roche). Samples were prepared with 5 μg of protein (cell lysates and conditioned media) mixed with 6× SDS sample buffer under reducing conditions. Samples were boiled at 100°C for 5 min and then analysed by 5% SDS-PAGE. Membranes were blocked in PBS containing 5% normal goat serum and 1% BSA. The membranes were incubated with α-hTnC polyclonal antibody (1:500) and rabbit α-β-actin polyclonal antibody (1:500). After washing, the membranes were incubated with the appropriate HRP-conjugated secondary antibody.

RNA extraction and quantification of mRNA for αSMA, TnC, pro-inflammatory cytokines and growth factors

Total RNA was extracted by using Trizol reagent (Invitrogen, Tokyo, Japan). First-strand cDNA was synthesized by using a random nine-mer primer and ReverTra Ace at 30°C for 10 min, 42°C for 1 h, 99°C for 5 min and 4°C for 5 min. PCR amplification was performed by using ExTaq DNA polymerase. Primers used for PCR analysis are shown in Table 1. After an initial check, we selected 32 cycles for RT-PCR.

Rat intestinal cell line IEC-6 cell

The rat small intestinal cell line IEC-6 was obtained from RIKEN Cell Bank (RCB0993; Japan). Cells were cultured in DMEM with 10% FBS containing 1% penicillin- streptomycin at 37°C in 5% CO_2/95% O_2. The cells were grown and used for assays of wound healing and trans-well migration.

Wound healing assay

IEC-6 cells were used for the migration assay (Nagaharu *et al.*, 2011). To coat six-well plates with TnC (gift from Professor Moriaki Kusakabe, The University of Tokyo, Japan), plates were incubated with 40 μg of TnC per well for 1 h (control wells had no TnC). A suspension of IEC-6 cells (2×10^6 cells per well) was poured into the wells and cultured until sub-confluence. The sub-confluent cells were further incubated with TnC (20 $\mu g \cdot mL^{-1}$), TGF-β1 (10 $ng \cdot mL^{-1}$), or both TnC and TGF-β1 for 24 h (controls had neither TnC nor TGF-β1; Wako Pure Chemical Industries, Osaka, Japan). The confluent cell layer was scratched by a pipette tip and the medium was changed. Photographs (four fields per well) were taken, and TnC, TGF-β1 or TnC-TGF-β1 was added at the same concentration.

Trans-well migration assay

Trans-well chambers (8 μm pore size; BD Biosciences, Durham, NC, USA) were prepared by pre-coating with gelatine (1%). The upper membrane of gelatine pre-coated inserts was then coated with TnC (40 μg) for 1 h. IEC-6 cells (5×10^5) in 300 μL of DMEM were placed in the 24-well upper chamber of the cell culture insert and 800 μL of DMEM was added to the lower chamber. FBS (1%), TnC (40 $\mu g \cdot mL^{-1}$) and TGF-β1 (20 $ng \cdot mL^{-1}$) were added to the lower chamber and TnC (40 $\mu g \cdot mL^{-1}$) was added to the upper chamber for 8 h at 37°C in 5% CO_2/95% O_2. Cells that migrated to the reverse side of the membranes were counted and photographed with a digital camera (Nikon 1200C; Nikon).

Statistical analysis

All experiments were performed at least in triplicate, and values are expressed as mean ± SEM. Statistical significance ($P < 0.05$, $P < 0.01$) was determined by using the Student's *t*-test or one-way ANOVA, as appropriate (Figures 1D and 9A, panel b).

Results

TnC expression was up-regulated in inflamed intestinal tissue

In control mice, TnC was densely distributed in muscle layers of colonic tissue (Figure 1). In the mucosal layer of control mice, only a small amount of TnC was detected in the submucosa at a subsurface epithelial region in the colon. On the other hand, in the inflamed colon tissue of DSS- (Figure 1) and TNBS-treated (Figure 2) mice, the immunohistochemical analyses showed dense TnC expression in the areas of tissue damage or inflammation. DSS-induced colitis exhibited significantly increased TnC mRNA levels on day 3 of DSS administration. The mRNA for TnC decreased on day 8, but still remained higher than the untreated control level ($P < 0.05$) (Figure 1D).

SAMP1/Yit mice exhibited a spontaneous gastrointestinal inflammation and provides a model remarkably similar to human Crohn's disease (Matsumoto *et al.*, 1998). As shown in Figure 3A, panel a, morphological changes such as villous atrophy and crypt hyperplasia were observed in ileum, but no such indications were observed in colon (Figure 3B, panel a)

Table 1

Sequence of the primers used for RT-PCR analysis

Primer sets	Orientation	Sequence (5′ to 3′)	PCR product
Mouse -TNF-α (NM_013693)	Forward	ACGGCATGGATCTCAAAGAC	324 bp
	Reverse	CGGACTCCGCAAAGTCTA AG	
Mouse-IL-1β (NM_008361)	Forward	TGACGTTCCCATTAGACAGC	497 bp
	Reverse	TGGGGAAGGCATTAGAAACA	
Mouse-IL-6 (NM_031168)	Forward	TCTCTGGGAAATCGTGGAAA	397 bp
	Reverse	GATGGTCTTGGTCCTTAGCC	
Mouse-IL-33	Forward	ATGAGACCTAGAATGAAGTATTCCA	(Hayakawa *et al.*, 2007) 801 bp
	Reverse	TTAGATTTTCGAGAGCTTAAACATA	
Mouse-IL-10 (NM_010548.2)	Forward	TGCTGCCTGCTCTTACTGAC	400 bp
	Reverse	GCTCCACTGCCTTGCTCTTA	
Mouse TnC	Forward	ACCATGCTGAGATAGATGTTCCAAA	(Meuronen *et al.*, 2011)
	Reverse	CTTGACAGCAGAAACACCAATCC	
Mouse-TGF-β1	Forward	TAGGAAGGACCTGGGTTGGAAG	(Murata *et al.*, 2011)
	Reverse	CGGGTTGTGTTGGTTGTAGAGG	
Mouse-PDGF-B (NM_011057.3)	Forward	TGAAATGCTGAGCGACCAC	(Chen *et al.*, 2006) 137 bp
	Reverse	AGCTTTCCAACTCGACTCC	
Mouse-GAPDH (M32599)	Forward	TGTTCCTACCCCCAATGTGT	269 bp
	Reverse	CCCTGTTGCTGTAGCCGTAT	
Rat-αSMA (NM_031004)	Forward	GGGAGTGATGGTTGGAATGG	197 bp
	Reverse	CCGTTAGCAAGGTCGGATG	
Rat-vimentin (NM_031140)	Forward	ACGAATACCGGAGACAGGTG	265 bp
	Reverse	TCCAGCAGCCTTCCTGTAGGT	
Rat-desmin (NM_022531)	Forward	ACCTGCGAGATTGATGCTCT	275 bp
	Reverse	AAGGTCTGGATCGGAAGGTT	
Rat-TnC	Forward	ATGTTGAATGGCGACAC	(Schermuly *et al.*, 2005)
	Reverse	CGGTCTCCAAACCCAG	
Rat-GAPDH (XM_576394)	Forward	TCCCTCAAGATTGTCAGCAA	308 bp
	Reverse	AGATCCACAACGGATACATT	

in SAMP1/Yit mice (original colony developed in Yakult Central Institute for Microbiological Research, Japan). In the inflamed ileal tissues of SAMP1/Yit mice, we also found a significant ($P < 0.01$) increase in TnC expression after 20 weeks of age, compared with age-matched control AKR mice (Figure 3A, panel d). On the contrary, both the SAMP1/Yit and AKR (control) mice showed only a basal level of TnC in the non-inflamed colon tissues (Figure 3B, panels b–d). These findings also indicate a close correlation between TnC production and inflammation.

Colon mucosa comprises a mixture of cells of different lineages including epithelial cells, lymphocytes, resident macrophages, subepithelial fibroblasts and fibroblast-like cells. Therefore, it is important to determine the specific type of cells that are responsible for the constitutive and pathological expression of TnC in colon mucosa. Because it is well established that TnC is mainly produced by mesenchymal cells, such as smooth muscle cells, fibroblasts and myofibroblasts, we focused on the possible contribution of ISEMF as TnC-producing cells. In control tissues, double staining with TnC and αSMA indicated that there was no overlap in their expression. However, in severely inflamed areas, TnC expression strongly overlapped with αSMA expression (Figure 1).

TnC-producing cells in colon tissues

Because TnC is an extracellular matrix protein, it is possible that TnC is secreted from cells as soon as it is produced. In the TnC knock-out mice created by targeted allele disruption, most of exon 2 in the TnC gene is deleted and the *E. coli* β-gal gene (*LacZ*) is inserted. Therefore, we next analysed TnC promoter activity in TnC knock-out mice by performing X-gal staining to detect β-gal and also β-gal antibody in the colon. The β-gal/αSMA and X-gal/αSMA staining in the colon tissues of TnC(–/–) mice suggested that αSMA-positive ISEMF constitutively produced TnC (Figure 4A).

TnC is controlled by intracellular signals via specific cell-surface receptors that provide a wealth of information regarding the complex nature of the extracellular matrix in development and disease (Jones and Jones, 2000b). Growth factors such as PDGF and TGF-β induce TnC gene expression

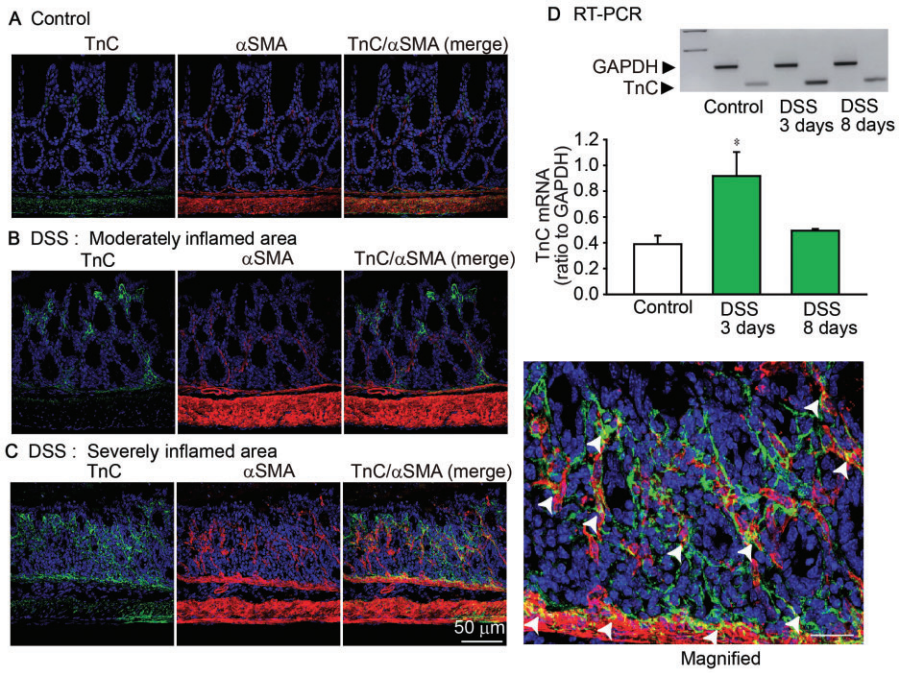

Figure 1

Increased TnC expression in mice with DSS-induced colitis. Experimental colitis was induced by 1% DSS in drinking water provided *ad libitum* for 8 days. Immunofluorescent staining of mid-colon tissue for TnC, αSMA and TnC/αSMA (merge) in (A) untreated control, (B) moderately inflamed areas of DSS-induced colitis and (C) severely inflamed areas of DSS-induced colitis. Bar = 50 μm. (D) Changes in TnC mRNA expression in the colons of mice with DSS-induced colitis (3–8 days). *$P < 0.05$, significantly different from control. $n = 4$–5.

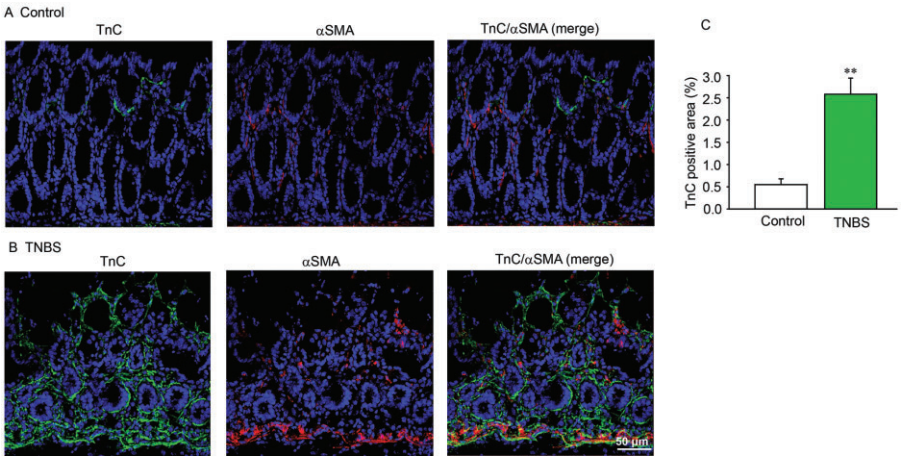

Figure 2

Increased TnC expression in mice with TNBS-induced colitis. Experimental colitis was induced by intrarectal administration of TNBS in 50% ethanol 150 mg·kg^{-1} bwt and mice were killed 2 days after TNBS administration. Immunofluorescent staining of mid-colon tissue for TnC, αSMA and TnC/αSMA (merge) in whole colon section of (A) control and (B) TNBS colitis. Bar = 50 μm. (C) Histogram showing significantly increased TnC-positive areas in TNBS colitis mice. **$P < 0.01$, significantly different from control. $n = 4$ each.

via classical signalling pathways as well as in part by stimulation of Rho/ROCK-dependent actin contractility (Chiquet *et al.*, 2004). In addition, PDGF-BB up-regulates TnC expression in rat aortic smooth muscle cells, and this up-regulation de-stabilizes the cell-matrix interaction and influences cell migration (LaFleur *et al.*, 1994; Wallner *et al.*, 2002). We then examined and characterized colonic TnC-positive ISEMF by TnC/PDGFRα and X-gal/PDGFRα staining in wild-type and knock-out mice respectively. The TnC-positive ISEMF were co-stained with PDGFRα (Figure 4B). Consistent with the *in vivo* results, ISEMF isolated from the rat colon expressed a constitutive level of TnC and were co-stained with PDGFRα

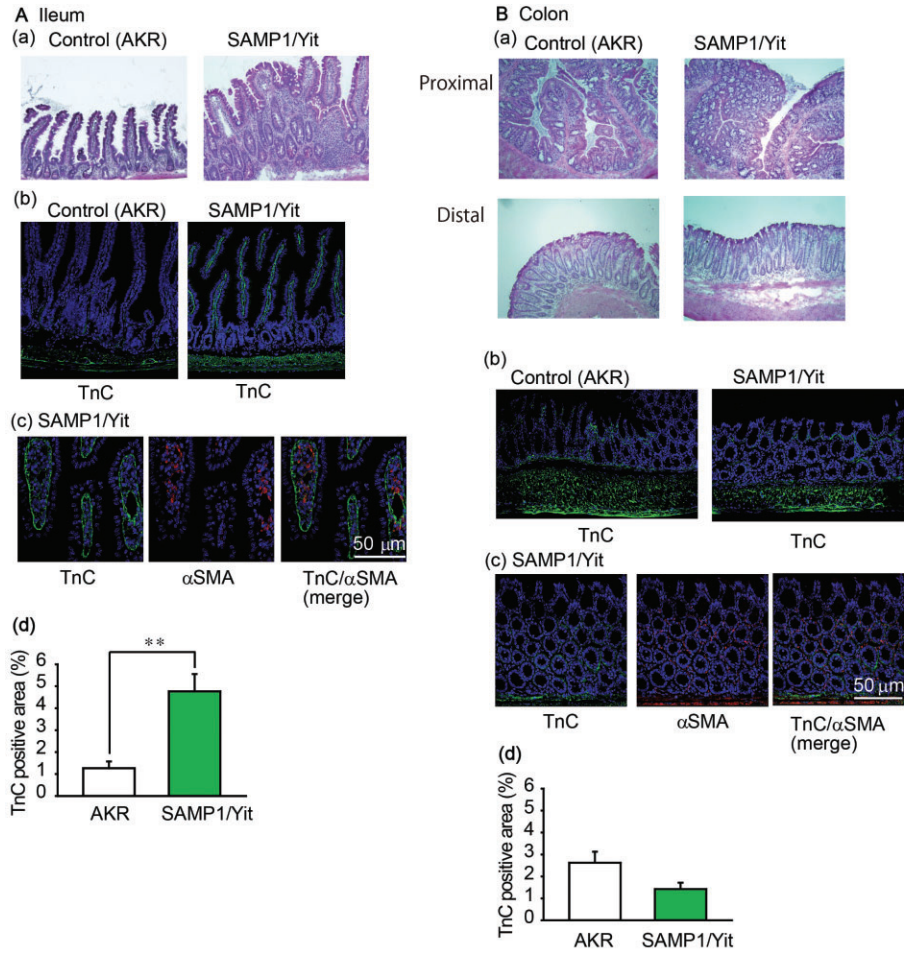

Figure 3

Histopathology and increased TnC expressions in SAMP1/Yit mice, a model of ileitis. (A, panel a) haematoxylin and eosin staining showing morphological changes of villous atrophy and crypts hyperplasia in SAMP1/Yit mice, compared with AKR mice. (A, panels b, c) Immunohistochemistry shows increased expression of TnC and (panel d) histogram shows significantly increased TnC-positive areas in the terminal ilea of SAMP1/Yit mice. $^{**}P < 0.01$, significantly different from AKR (control). $n = 3–4$. Bar = 50 μm. (B, panel a) HE staining shows no morphological changes of colon (proximal and distal), (panels b, c) immunohistochemistry shows basal TnC expression and (panel d) histogram shows TnC-positive areas in the colon of AKR and SAMP1/Yit mice. $n = 3–4$. Bar = 50 μm.

(Supporting Information Fig. S1). Our immune-electron microscopic analysis confirmed that TnC-producing cells were present just beneath the colonic epithelia (Figure 4C). The localization and morphological characteristics indicated that these immunoreactive cells could be ISEMF.

DSS-induced colitis increases mRNA of pro-inflammatory cytokines and growth factors

We investigated DSS-induced colitis in mice with a C57BL/6N genetic background (Figure 5A–C). A combinatorial disease activity index calculated from the levels of body weight loss, stool consistency and stool blood was used to assess the effects of DSS-induced colitis. DSS-treated mice developed diarrhoea that started 4–5 days after DSS administration. Histological examination of the colons from mice given DSS in drinking water showed mucosal inflammation involving all layers of the bowel wall in some areas. Extensive granulation tissues with the presence of monocytes and lymphocytes and depletion of goblet cells were apparent in the mucosa.

We then examined mRNA level of pro-inflammatory cytokines and growth factors in the colitis tissues compared with untreated colitis mice. The changes in mRNA expression of IL-1β, IL-6, IL-10, IL-33, TNF-α, TGF-β1 and PDGF-B in colons (relative ratio to GAPDH) are shown in Figure 5D, panels a, b. Our results demonstrated that the expression of these inflammatory cytokines IL-1β, IL-6, IL-10, IL-33, TNF-α, and growth factors TGF-β1 and PDGF-B was significantly ($P < 0.05$ and $P < 0.01$) greater in DSS-treated mice.

PDGF-BB and TGF-β1 stimulated TnC expression in ISEMF

Next, we further analysed TnC up-regulation, excretion and/or deposition. Immunohistochemical studies demonstrated that the treatment of cells with PDGF-BB (20 ng) for 24 h caused a striking increase in TnC expression, and

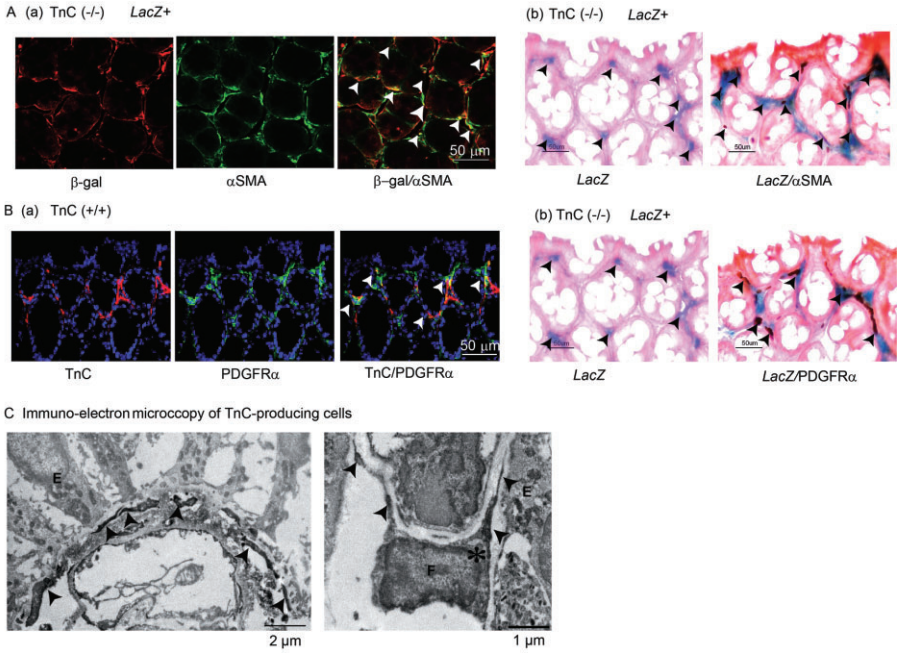

Figure 4

Identification and characterization of TnC-producing cells in the mid-colon of mice. (A, panel a) Immunofluorescent expression of β-gal, αSMA and their overlay (arrows) in *LacZ*-inserted TnC(–/–) mice. (A, panel b) X-gal staining showing *LacZ*- and *LacZ*/α-SMA-positive cells around the crypts of the colon in TnC(–/–) mice. Arrows indicate the overlay and co-localization of TnC-producing cells. (B, panel a) Immunofluorescent staining of TnC, PDGFRα and their overlays (arrows) in the mid-colon of C57BL/6N mice. (B, panel b) X-gal staining detected *LacZ*-positive and *LacZ*/ PDGFRα double-positive cells (arrows) in TnC(–/–) mice. n = 5–6. Bar = 50 μm. (C) Immunoelectron photograph showing the TnC immunoreactive cells in the distal colon of Balb/c mice. Left: TnC immunoreactive cells and their processes (arrows) were located in the apical portion of the lamina propria and just beneath the epithelia (E). Right: TnC immunoreactive cell (F). Immunoreactive deposits were mainly observed in the thin processes (arrows). Endoplasmic reticulum (*) was conspicuous in the cytoplasm. Bar = 1–2 μm.

Western blot analysis confirmed that PDGF-BB significantly increased TnC protein levels in the conditioned medium (P < 0.05) and cell lysates (P < 0.01) (Figure 6). Note that our antibody detected two bands of imunnoreactive TnC in Western blot analysis, both in cell lysate and in conditioned medium. The molecular weight markers indicated that the larger isoform of TnC (L-Tnc) was about 200 kDa and the smaller isoform of TnC (S-TnC) was about 180 kDa. In ISEMF, both the isoforms were increased in activated myofibroblasts. Phase-contrast microscopy before and after treatment indicated that PDGF-BB caused robust proliferation of the ISEMF cells, and RT-PCR analysis confirmed that this growth factor significantly increased the TnC mRNA level in ISEMF.

TGF-β1 is an important cytokine secreted from most immune cells and it promotes many cellular functions including cell growth, proliferation, differentiation and apoptosis. We found that TGF-β1 (5 ng·mL^{-1}) stimulation for 24 h increased TnC (both L-TnC and S-TnC) expression, but the increased TnC may be secreted by cells into the medium, as confirmed by Western blot analysis of conditioned medium and cell lysates (Figure 7). This finding indicated that a different mechanism underlying the excretion of TnC is controlled by TGF-β1, compared with that induced by PDGF-BB. RT-PCR analysis revealed that TGF-β1 increased the level of TnC mRNA, although this effect was not statistically significant.

We also investigated the effects of 24 h stimulation with IL-1β and TNF-α on rat ISEMF. But these treatments did not significantly up-regulate the TnC mRNA or protein levels in the conditioned medium or cell lysates (Supporting Information Figs S2, S3).

TnC gene deletion aggravated DSS-induced acute colitis in mice

We investigated the effects of TnC gene deletion on DSS-induced colitis in mice with a C57BL/6N genetic background (Figure 8A–D), assessing the severity of the colitis as described above. Both TnC(+/+) and TnC(–/–) littermates developed DSS-induced colitis but the TnC(–/–) mice exhibited more severe colitis than their TnC(+/+) littermates. TnC(+/+) mice developed diarrhoea that started 4–5 days after DSS administration, but there was no macroscopically visible blood in the stool even on the day of killing. On the contrary, TnC(–/–) littermate mice had bloody stools starting from day 4 to day 5 of DSS administration through the day of killing.

Histological examination of the colons of TnC(+/+) and TnC(–/–) littermates given DSS in drinking water showed mucosal inflammation involving all layers of the bowel wall in some areas. Extensive granulation tissues with the presence of monocytes and lymphocytes and depletion of goblet cells were apparent in the mucosa. In all experiments, TnC(–/–) mice exhibited more severe disease than TnC(+/+) littermates.

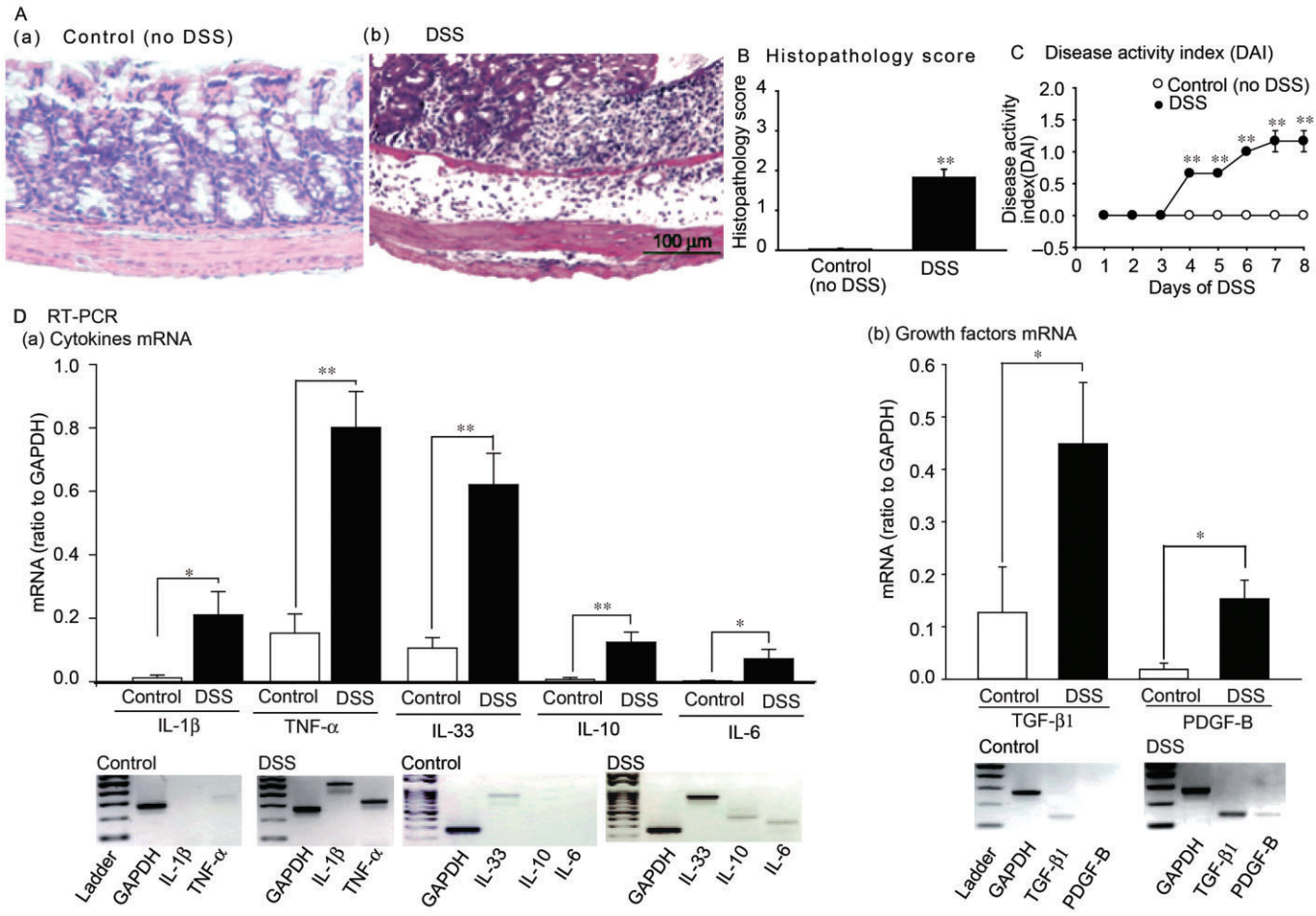

Figure 5

DSS-induced colitis increased mRNA level of pro-inflammatory cytokines and growth factors. Experimental colitis was induced by 1% DSS in drinking water provided *ad libitum* for 8 days to C57BL/6N mice. (A) Histology of full-thickness sections of mid-colon in (A, panel a) control: no DSS and (b) DSS. $n = 4–6$. Bar = 100 μm. (B) Histopathology score. $^{**}P < 0.01$, significantly different from control. $n = 4–6$. (C) Disease activity index. $^{**}P < 0.01$, significantly different as indicated. (D) RT-PCR analyses for changes in (panel a) IL-1β, TNF-α; IL-33, IL-10, IL-6; (panel b) TGF-β1, PDGF-B mRNA expression in DSS-induced (8 days) colitis mice. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different as indicated.

To confirm the effect of TnC deletion, we studied TnC-deleted mice on a Balb/c background (Figure 8E–F). Compared with C57BL/6N mice, the Balb/c strain was less sensitive to DSS. Treatment with 2% DSS for 5 days induced only moderate mucosal inflammation without the mucosal loss of intestinal epithelial cells (IEC) in TnC(+/+) mice. DSS-treated TnC(–/–) mice, on the other hand, demonstrated severe mucosal loss in the colon tissues as compared with the wild-type DSS-treated mice.

TnC enhanced cell migration

TnC produced by ISEMF cells may have some biological activity. Because TnC deletion exhibited severe mucosal damage, we then investigated trans-well migration using the IEC-6 rat enterocyte cell line. Our trans-well migration assay demonstrated strong chemotactic activity induced by FBS (1%) and moderate chemotactic activity induced by TGF-β1 (20 ng·mL^{-1}), whereas TnC (40 ng·mL^{-1}) exhibited only low levels of chemokinesis rather than chemotaxis. Interestingly, a significant number of IEC-6 cells migrated across the trans-well membrane when coated with TnC (40 μg·mL^{-1}) encased in gelatine, compared with gelatine alone. This observation indicated that TnC was a permissive substrate for epithelial cell invasiveness in the trans-well migration assay (Figure 9A, panels a, b).

We then performed a wound healing assay. The TnC-treated cells (20 μg·mL^{-1}) after 18 h showed enhanced cell migration towards the wound edges, compared with the untreated control cells. Cell migration towards the wound edges was strongly enhanced by TGF-β1 (10 ng·mL^{-1}) and even more strongly enhanced by combined treatment with TGF-β1 and TnC (Figure 9B, panels a, b).

Discussion

Among the animal models of inflammatory bowel disease, the oral administration of DSS has been widely used to study

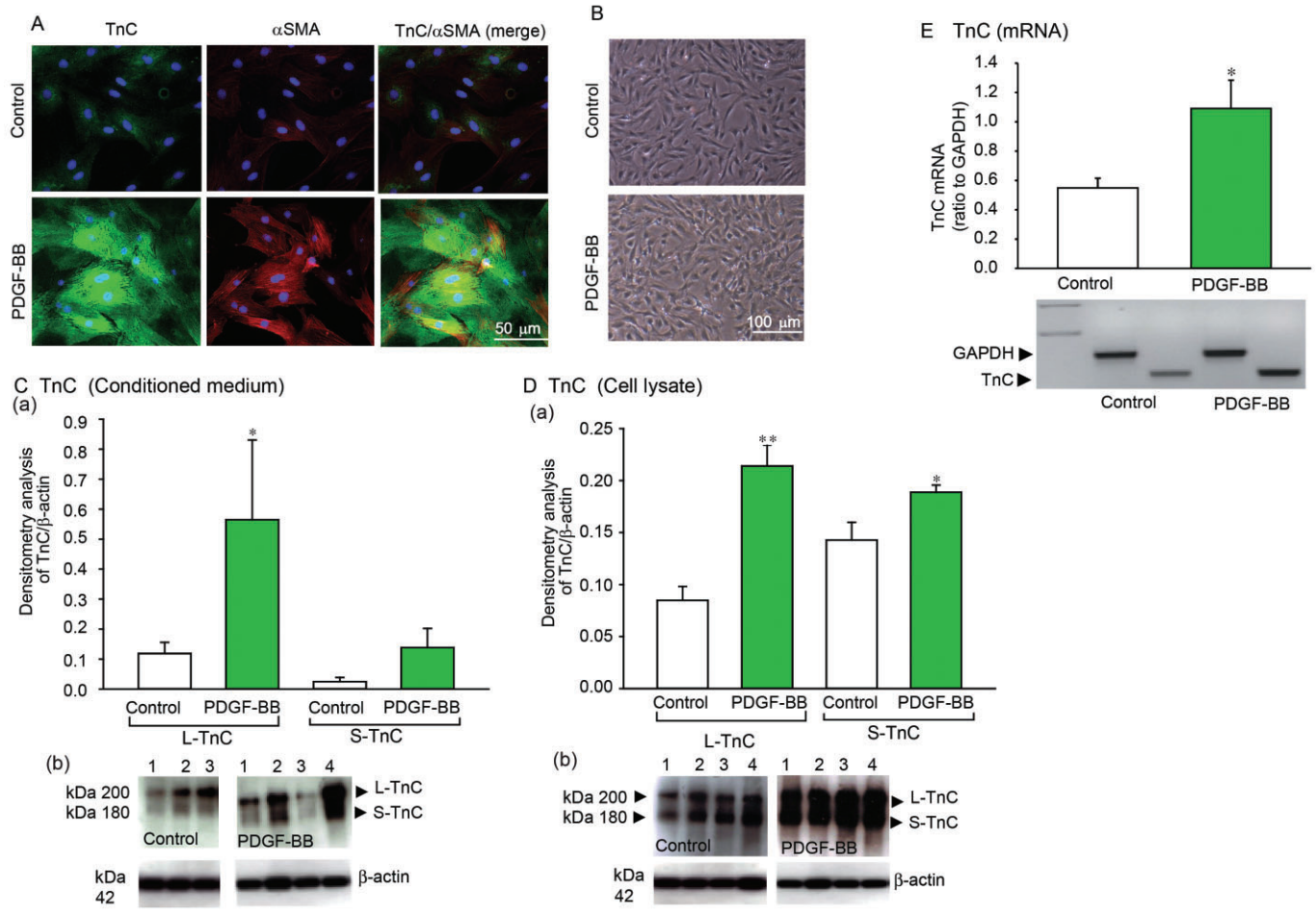

Figure 6

PDGF-BB up-regulates TnC in cultured rat ISEMF. ISEMF were stimulated with PDGF-BB (20 ng·mL^{-1}) for 24 h. (A) Immunofluorescent histopathology of TnC and αSMA and TnC/αSMA overlaps. PDGF-BB increased the expression of both TnC and αSMA. Bar = 50 μm; n = 4–5. (B) Phase-contrast microscopy showing morphological changes of rat ISEMF caused by PDGF-BB. n = 4–5. Bar = 100 μm. (C–D) Western blot analyses of TnC in conditioned medium and cell lysates respectively. (panel a) The upper histogram is the data derived from Western blots following densitometry analysis. (panel b) The lower panel shows an example of Western blot following probing with the antibody for TnC. *P < 0.05, **P < 0.01, significantly different from control. (E) RT-PCR analysis of TnC mRNA expression in PDGF-BB-treated rat primary ISEMF. *P < 0.05, significantly different from control. n = 4–5.

the mechanisms of colonic inflammation (Aharoni *et al.*, 2006). In this study, immunohistochemical analysis showed dense TnC expression in areas of tissue damage or inflammation after administration of DSS or of TNBS, and DSS-induced colitis also increased expression of mRNA for TnC. Because mouse models of spontaneous disease are believed to provide more sophisticated pathophysiological information than induced disease models, we also used SAMP1/Yit mice, a model of Crohn's disease, to determine the profile of TnC appearance during ileal inflammation. *In vivo* investigations with this ileitis model demonstrated that TnC was highly expressed in the damaged ileal mucosal region of intestinal inflammation. Additionally, immunohistochemistry, *LacZ* assays and immune electron microscopy demonstrated that the TnC-producing cells in the mucosa were ISEMF, and that these cells expressed PDGFRα.

We further observed that in tissues from mice with DSS-induced colitis, there was a significant increase of growth factors, such as PDGF-B and TGF-β1, as well as cytokines such as IL-1β, IL-6, IL-10, IL-33 and TNF-α. *In vitro* studies of isolated ISEMF suggested that PDGF-BB profoundly up-regulated TnC. Western blot analyses of cell lysates and conditioned medium revealed that the up-regulated TnC protein was released into the medium but that a significant quantity remained inside the cells. Another growth factor, TGF-β1, also strongly up-regulated TnC levels in cultured ISEMF, and Western blot analyses confirmed that the up-regulated TnC was immediately released from cells into the surrounding medium. Thus, although both growth factors PDGF-BB and TGF-β1 stimulated and up-regulated TnC, the profiles of cellular excretion were different.

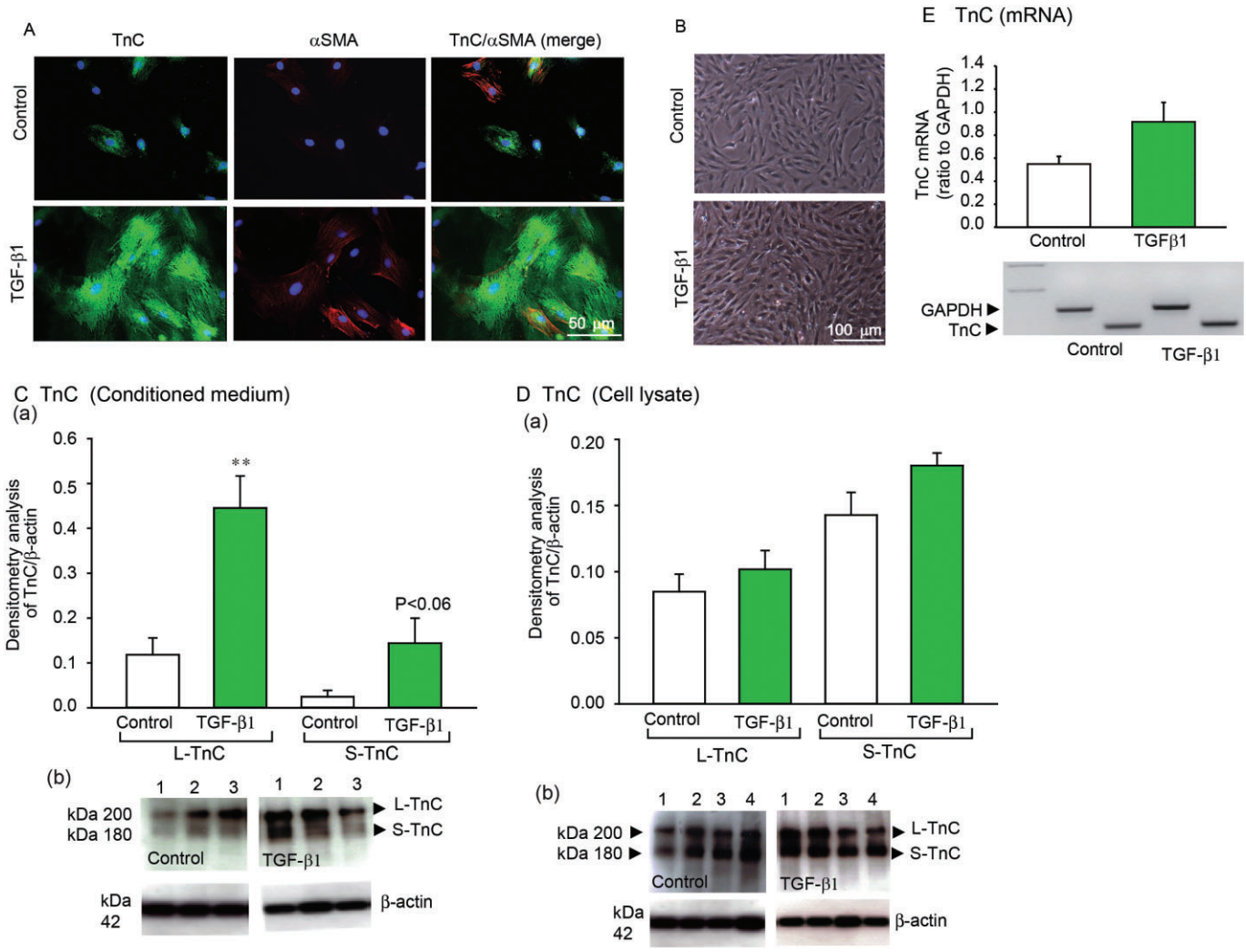

Figure 7

TGF-β1 up-regulates TnC in cultured rat ISEMF. ISEMF were stimulated with TGF-β1 (5 ng·mL^{-1}) for 24 h. (A) Immunofluorescent histopathology of TnC and αSMA and TnC/αSMA overlaps. TGF-β1 increased the expression of both TnC and αSMA. Bar = 50 μm; n = 4–5. (B) Phase-contrast microscopy showing morphology of rat ISEMF treated with TGF-β1. n = 4–5. Bar = 100 μm. (C–D) Western blot analyses of TnC in conditioned medium and cell lysates respectively. (panel a) The upper histogram is the data derived from Western blots following densitometry analysis. (panel b) The lower panel shows an example of Western blot following probing with the antibody for TnC. **P < 0.01, significantly different from control. (E) RT-PCR analysis of TnC mRNA expression in TGF-β1-treated rat primary ISEMF. n = 4–5.

Next, we examined the role of TnC using TnC null mice. DSS-induced colitis occurred in both TnC(+/+) and TnC(–/–) littermates; however, the inflammation was greater in TnC(–/–) mice. Disease severity, neutrophil infiltration, macrophages/monocytes and mRNA levels of pro-inflammatory cytokines IL-1β and TNF-α were relatively higher in TnC(–/–) mice. TnC(–/–)mice with DSS-induced colitis exhibited fecal blood which increased in severity until killing, whereas TnC(+/+) mice with DSS-induced colitis developed diarrhoea with little fecal blood. TnC(–/–) mice exhibited a loss of surface epithelia that may have resulted in blood oozing into the feces. These results suggest that TnC may have the potential to reduce acute intestinal inflammation by accelerating mucosal barrier repair or protection. Interestingly, the increase of inflammatory parameters seemed to be less than that of real disease activity (data not shown), indicating that TnC primarily affects mucosal repair processes rather than directly inhibiting pro-inflammatory processes.

It is notable that TnC-deficient mice exhibited severe mucosal damage. Cell migration is an important process in wound healing and the migrating cells are known to interact with the extracellular matrix, which provides a suitable environment for inflammation followed by repair. Our *in vitro* study on cell migration revealed that the extracellular addition of TnC enhances the migration of IEC-6 cells. Additionally, TnC significantly enhanced cell migration when the

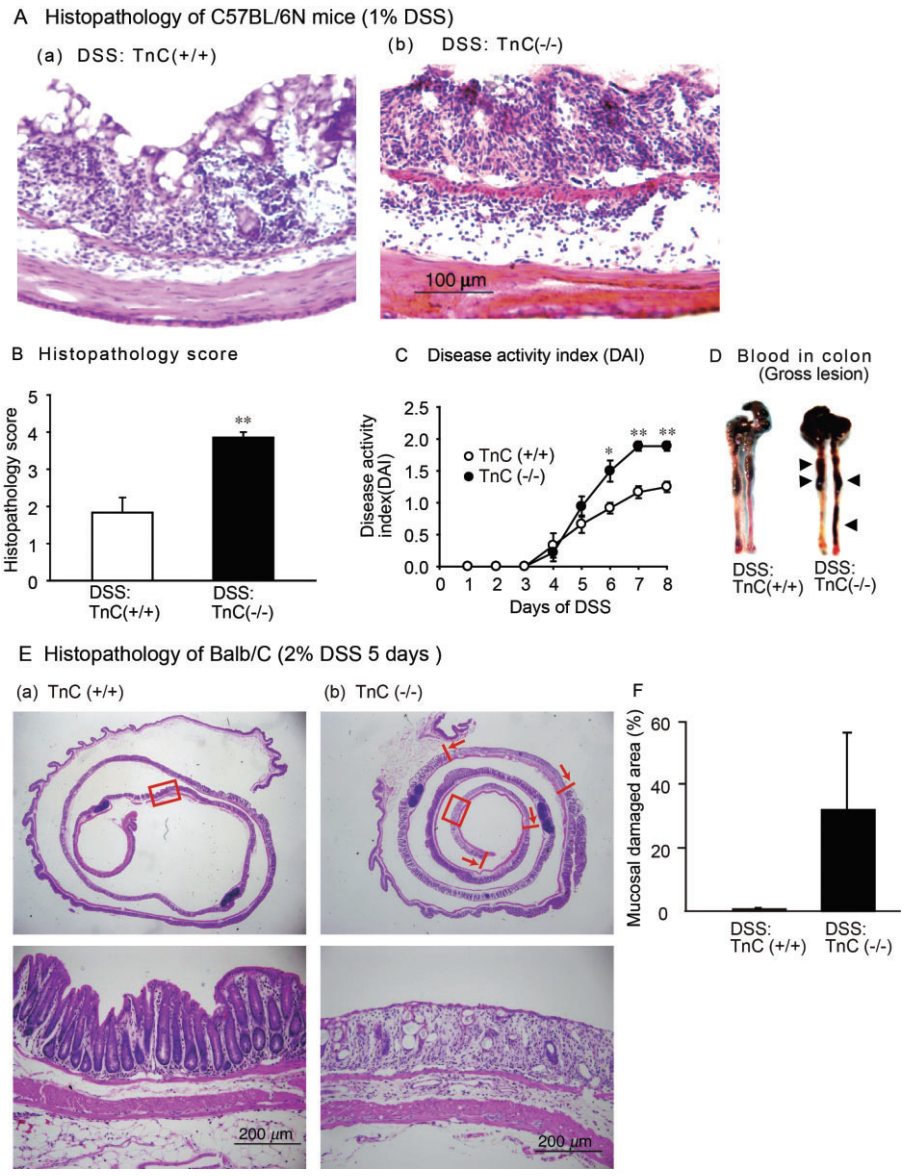

Figure 8

Effects of TnC gene deletion in DSS-induced colitis. Experimental colitis was induced by 1% DSS in drinking water provided *ad libitum* for 8 days to C57BL/6N mice. (A) Histology of full-thickness sections of mid-colon in (panel a) DSS: TnC(+/+) and (panel b) DSS: TnC(–/–). n = 4–6. Bar = 100 μm. (B) Histopathology score. $^{**}P < 0.01$, significantly different from TnC(+/+). n = 4–6. (C) Disease activity index. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from TnC(+/+). (D) Representative photographs of bloody feces in the colon. Arrows indicate the bloody feces in the colon of TnC(–/–) mice. n = 4–6. (E) Experimental colitis was induced by 2% DSS in drinking water provided *ad libitum* for 5 days to Balb/C mice. Photographs represent intestinal mucosal inflammation in TnC(+/+) mice (panel a, left) and TnC(–/–) mice (panel b, right). The upper panel shows the length of intestine, and the respective damage areas are indicated by arrows. Lower panels are shown with high magnification of the boxed areas. (F) Analytical data for intestinal mucosal damaged areas (%) of intestine in TnC(+/+) and TnC(–/–) mice. n = 3 each. Bar = 200 μm.

upper membrane of the trans-well chamber was coated with TnC encased in gelatine as compared with gelatine alone. TnC also demonstrated moderate chemokine activity. TnC inherently acts as a permissive substrate for invasive cells (Deryugina and Bourdon, 1996; Sarkar *et al.*, 2006). Therefore, TnC may play vital roles in wound repair of mucosal tissues.

The signalling molecules that regulate TnC homeostasis seem to be complex because TnC is a multi-domain molecule and it participates in both pro-inflammatory and anti-inflammatory signalling cascades. TnC mediated pro-inflammatory activity by T-cell stimulation (Nakahara *et al.*, 2006; El-Karef *et al.*, 2007) and increased production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 via

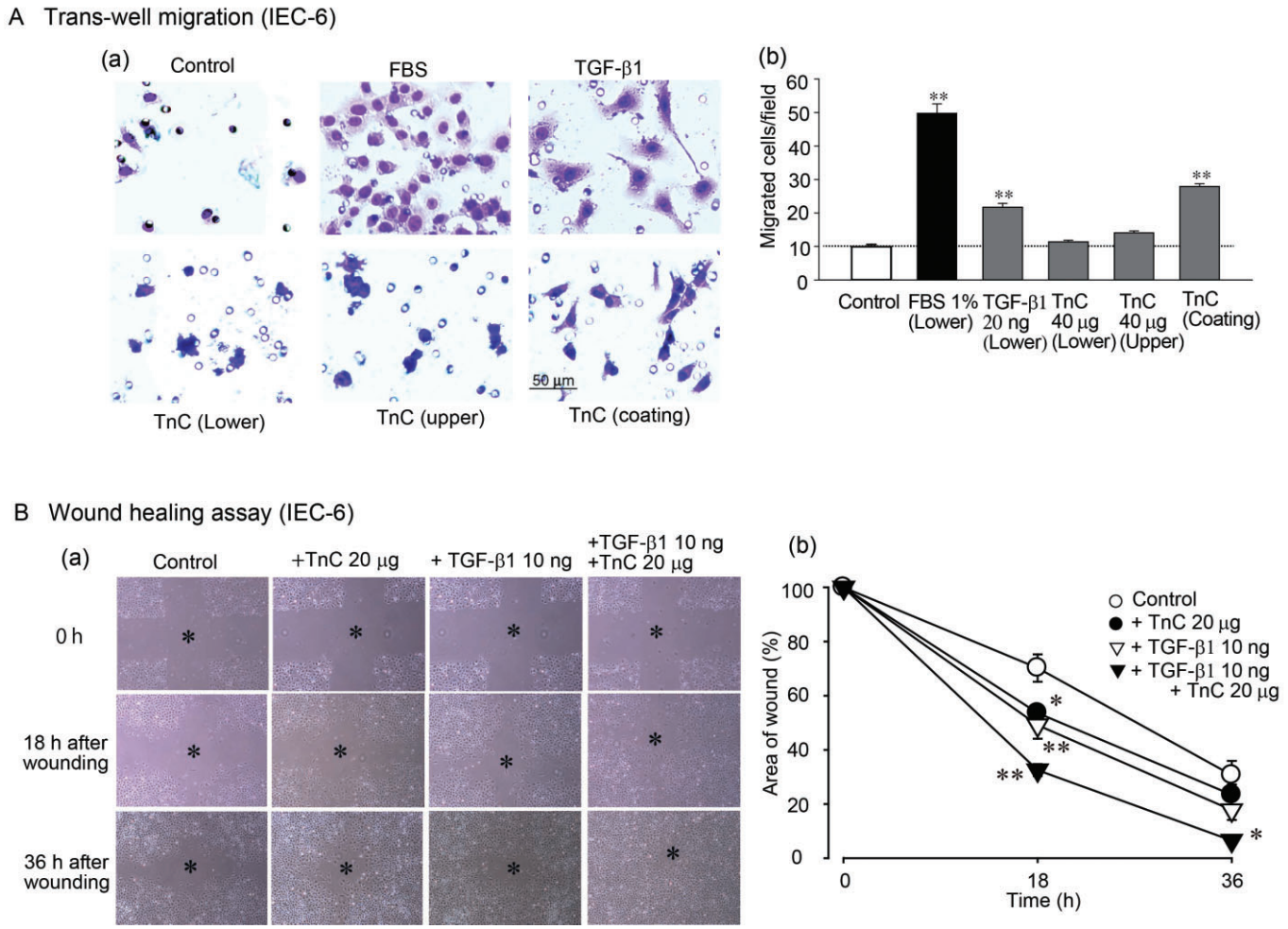

Figure 9

TnC enhances IEC cell migration. TnC coating increases IEC-6 cell migration through the trans-well membrane. (A) IEC-6 cells were allowed 8 h to migrate through trans-wells membranes (panel a), control (no treatment), FBS 1% (lower), TGF-β1 20 ng (lower), TnC 40 μg (lower), TnC 40 μg (upper) and TnC 40 μg (coating). Bar = 50 μm. (A, panel b) Analytical data showing increased migration of cells at 8 h of FBS, TGF-β1 and TnC coating treatment. $^{**}P < 0.01$, significantly different from control. $n = 4$ each. (B) IEC-6 cells treated with TnC (20 μg·mL^{-1}) enhanced migration towards wound edges. (panel a) Photographs showing control and treated (TnC, TGF-β1 and TnC + TGF-β1) cells at 0, 18 and 36 h. (panel b) Analytical data showing increased migration of treated cells at 18 h. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control. $n = 5$ each.

TLR4 receptor stimulation (Midwood *et al.*, 2009). Anti-inflammatory effects of TnC were mediated through the inhibition of T-cell activation (Ruegg *et al.*, 1989) and increased production of the anti-inflammatory cytokine IL-4 (Makhluf *et al.*, 1996). Deletion of the TnC gene exacerbated spinal cord injury (Chen *et al.*, 2010), glomerulonephritis (Nakao *et al.*, 1998), atherosclerosis (Wang *et al.*, 2012) and infiltration of polymorphonuclear cells (Koyama *et al.*, 1998) as compared with wild-type mice.

In summary, TnC homeostasis was controlled by growth factors such as PDGF and TGF-β in colonic tissues (Figure 10). PDGF and TGF-β increased TnC production through some signalling pathways on ISEMF. TnC accelerated cell migration and remodelling, which are involved in reparative processes in the damaged areas. Deletion of the TnC gene exacerbated DSS-induced colitis. These findings disclose protective actions of TnC in DSS-induced acute colitis and suggest that TnC signalling associated with PDGF and TGF-β plays important pharmacological roles during inflammation.

Acknowledgements

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Conflict of interest

There are none to declare.

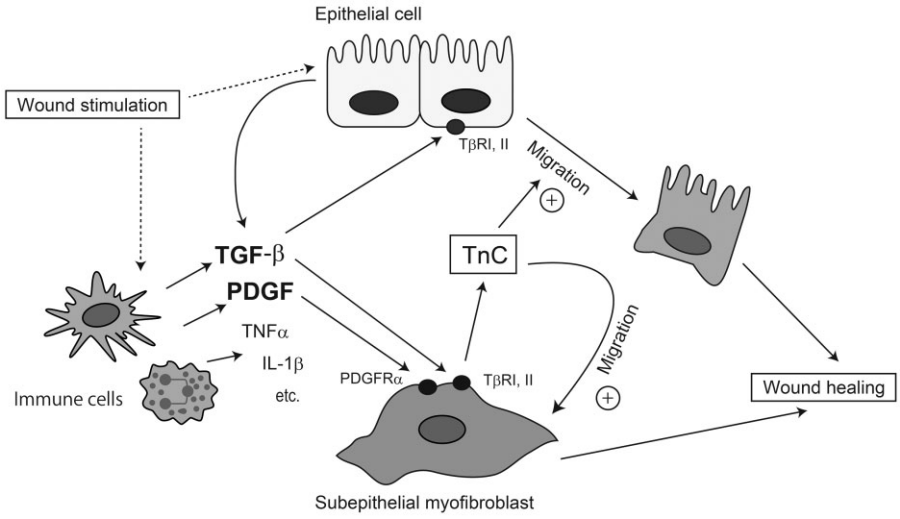

Figure 10

Schematic representations of the roles of TnC in the amelioration of intestinal inflammation. Wound stimulation or inflammation triggers immune cells to produce growth factors such as PDGF and TGF-β. PDGF and TGF-β then activate ISEMF causing increased production of TnC. Increased TnC protects the mucosal barrier as well as accelerating cell migration and cellular remodelling to repair the damaged areas.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12452

Figure S1 Rat ISEMF constitutively produces TnC. (A) Immunofluorescent staining of TnC, αSMA, TnC/αSMA (merge) (upper panel); TnC, PDGFRα, TnC/ PDGFRα (middle panel); vimentin and desmin (lower panel). Nuclei were stained with DAPI. Bar = 20 μm. (B) RT-PCR analysis of ISEMF. ISEMF expressed mRNA for αSMA (196 bp), vimentin (265 bp), but not desmin (275 bp). GAPDH (308 bp). n = 5–6.
Figure S2 IL-1β did not significantly up-regulate TnC in cultures of rat ISEMF. ISEMF cells were cultured and starved for 24 h without giving FBS followed by stimulation with IL-1β (10 ng·mL^{-1}) for 24 h. (A–B) Western blotting analysis of TnC in the conditioned medium and cell lysates respectively. (A) The upper histogram is the data derived from Western blots following densitometry analysis. (B) The lower panel shows an example of Western blot following probing the antibody for TnC. (C) RT-PCR analysis of TnC mRNA is shown. No significant differences were observed in protein and mRNA level of TnC. n = 4 each. L-TnC, large isoform of tenascin-C; S-TnC, small isoform of tenascin-C.
Figure S3 TNF-α-stimulation did not significantly up-regulate TnC in cultures of rat ISEMF. (A–B) Western blotting analysis of TnC in the conditioned medium and cell lysates respectively. (A) The upper histogram is the data derived from Western blots following densitometry analysis. (b) The lower panel shows an example of Western blot following probing the antibody for TnC. (C) RT-PCR analysis of TnC mRNA is shown. No significant differences were observed in protein and mRNA level of TnC. n = 4 each. L-TnC, large isoform of tenascin-C; S-TnC, small isoform of tenascin-C.