

Constitutive TL1A Expression under Colitogenic Conditions Modulates the Severity and Location of Gut Mucosal Inflammation and Induces Fibrostenosis

Robert Barrett,* Xiaolan Zhang,*[†] Hon Wai Koon,[‡] Michelle Vu,* Jyh-Yau Chang,* Nicole Yeager,* Mary Ann Nguyen,* Kathrin S. Michelsen,* Dror Berel,* Charalabos Pothoulakis,[‡] Stephan R. Targan,* and David Q. Shih*

From the Inflammatory Bowel & Immunobiology Research Institute,* Cedars-Sinai Medical Center, Los Angeles, California; the Department of Gastroenterology,[†] The Second Hospital of Hebei Medical University, Hebei, China; and the IBD Center,[‡] David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California

Intestinal fibrostenosis is a hallmark of severe Crohn's disease and can lead to multiple surgeries. Patients with certain *TNFSF15* variants overexpress TL1A. The aim of this study was to determine the effect of TL1A overexpression on intestinal inflammation and the development of fibrostenosis. We assessed the *in vivo* consequences of constitutive TL1A expression on gut mucosal inflammation and fibrostenosis using two murine models of chronic colitis. In the dextran sodium sulfate (DSS) and adoptive T-cell transfer models, there was proximal migration of colonic inflammation, worsened patchy intestinal inflammation, and long gross intestinal strictures in *Tll1a* transgenic compared to wild-type littermates. In the DSS model, myeloid- and T-cell-expressing *Tll1a* transgenic mice had increased T-cell activation markers and interleukin-17 expression compared to wild-type mice. In the T-cell transfer model, *Rag1*^{-/-} mice receiving *Tll1a* transgenic T cells had increased interferon- γ expression but reduced T-helper 17 cells and IL-17 production. Narrowed ureters with hydronephrosis were found only in the *Tll1a* transgenic mice in all chronic colitis models. In human translational studies, Crohn's disease patients with higher peripheral TL1A expression also exhibited intestinal fibrostenosis and worsened ileocecal inflammation with relative sparing of rectosigmoid inflammation. These

data show that TL1A is an important cytokine that not only modulates the location and severity of mucosal inflammation, but also induces fibrostenosis. (Am J Pathol 2012, 180:636–649; DOI: 10.1016/j.ajpath.2011.10.026)

Crohn's disease (CD) is a chronic inflammatory condition with pathological features such as patchy transmural gut inflammation and fibrostenosis with relative sparing of the rectum.^{1,2} Fibrostenosis is a characteristic of severe, often treatment-resistant, disease, a source of significant morbidity, and in nearly every case, requires surgical intervention. Variants in the *TNFSF15* gene have been found to be associated with inflammatory bowel disease (IBD); and its protein product, TL1A, is elevated in the intestinal mucosa of IBD patients. Certain *TNFSF15* haplotypes are associated with susceptibility in non-Jewish Caucasian CD and UC patients. In addition, *TNFSF15* haplotype-B is not only associated with risk, but also with severity in Jewish CD patients.^{3–5} Moreover, monocytes from Jewish patients carrying the risk haplotype-B express higher peripheral monocyte TL1A expression in response to Fc γ R stimulation.³ These results show that CD-associated *TNFSF15* genetic variations contribute to enhanced induction of TL1A, resulting in severe, chronic mucosal inflammation- and that modulation of TL1A may be a potential target for therapeutic development.

TL1A signals via death domain receptor 3 (DR3) and modulates the adaptive immune response. In the T-helper (Th)-1 effector arm, TL1A enhances interferon (IFN)- γ production from peripheral and mucosal

Supported by U.S. Public Health Service grant DK056328.

Accepted for publication October 31, 2011.

R.B. and X.Z. contributed equally to this work.

Supplemental material for this article can be found at <http://ajp.amjpathol.org> or at doi: 10.1016/j.ajpath.2011.10.026.

Address reprint requests to David Q. Shih, M.D., Ph.D., Cedars-Sinai Inflammatory Bowel and Immunobiology Research Institute, 8700 Beverly Blvd., Suite 4066, Los Angeles, CA 90048. E-mail: david.shih@csmc.edu.

T cells.^{6,7} In addition to mediating the Th-1 response, TL1A also plays a role in augmenting Th-2 and Th-17 effector cell function.^{8–11} The differential effect of TL1A on Th-17 cells was demonstrated by a recent report showing that TL1A inhibited Th-17 development in naive T cells but maintained the effector characteristics of committed Th-17 cells.¹²

In addition to human reports, several studies in mice also implicate the TL1A/DR3 signaling pathway in mucosal inflammation.^{13,14} Neutralizing TL1A antibody ameliorates inflammation in DSS and *Gai2*^{-/-} T-cell transfer chronic colitis models.¹⁰ Additionally, constitutive TL1A expression in mice leads to mild spontaneous ileitis and increased collagen deposition.^{15–17}

To determine the *in vivo* consequences of constitutive TL1A expression under colitogenic settings, chronic dextran sodium sulfate (DSS)-induced [myeloid and T-cell *T11a* transgenic (Tg) mice] and adoptive-transfer (T-cell *T11a* transgenic mice) models were used. In all models, there was proximal migration of colonic inflammation, worsened patchy intestinal inflammation, and strictures in the intestine and colon with constitutive TL1A expression. Similarly, CD patients with *TNFSF15* haplotype-B and elevated TL1A expression in primary monocytes showed worsened ileocecal inflammation with relative rectosigmoid sparing and intestinal fibrostenosis. These novel murine *T11a*-Tg models resemble a complicated form of severe human CD. Results from these *T11a*-Tg experimental models coupled with findings in CD patients suggest that *TNFSF15* is a severity gene and that the cytokine, TL1A, when overexpressed, plays a central role in generating one phenotypic form of complicated CD.

Materials and Methods

Transgenic Mice and Induction of Chronic Colitis

LCK-CD2-T11a-GFP (L-Tg) and *FMS-T11a-GFP* Tg (M-Tg) mice were generated and genotyped as described.¹⁷ Chronic DSS colitis was induced as described,¹⁰ except 2% (w/v) DSS (MP Biomedicals, Irvine, CA) drinking water was used due to lethality in *T11a*-Tg mice when DSS concentration $\geq 2.5\%$ was used. In the adoptive-transfer model, colitis was induced by intraperitoneal injection of 500,000 CD4⁺CD45RB^{hi} naive T cells isolated from either L-Tg or wild-type (WT) mice to *Rag1*^{-/-} mice.¹⁸ All mice were maintained under specific pathogen-free conditions in the Animal Facility at Cedars-Sinai Medical Center (CSMC). Littermate control mice were used. This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal studies were approved by the CSMC Animal Care and Use Committee (protocol 2269).

Quantitation of DSS Water Consumption

WT, L-Tg, and M-Tg mice ($n = 5$ in each group) were housed individually in a cage and administered 2% DSS water on days 1 to 5, 8 to 12, 15 to 19, and 22 to 26. The

weight of DSS water was measured at the beginning of each cycle (days 1, 8, 15, and 22) and at the end of the cycle (days 5, 12, 19, and 26). DSS water consumption was measured by subtracting DSS water weight at the beginning of the treatment cycle from the end of the treatment cycle.

Disease Activity Index, Myeloperoxidase, and Macroscopic and Histopathological Analyses

The disease activity index (DAI) was calculated by scoring from 0 to 4 abnormalities regarding changes in body weight (0, no weight loss; 1, 1% to 5% weight loss; 2, 5% to 10% weight loss; 3, 10% to 15% weight loss; 4, more than 15% weight loss), stool consistency (0, firm dry stool; 1, moist stool; 2, soft adherent stool; 3, large soft pliable stool; 4, liquid stool), stool blood performed on Hemocult Sensa (Beckman Coulter, Brea, CA; 0, no color; 1, flecks of blue; 2, up to 50% blue; 3, more than 50% blue; 4, gross red blood), and summing the results.¹⁹ DAI was determined every other day for the DSS model and twice a week for the adoptive-transfer model. Myeloperoxidase activity was assessed using the EnzChek Myeloperoxidase Activity Assay Kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Macroscopic evidence of inflammation was scored blinded to the mice genotype using the established classification.^{10,18} Briefly, normal gut morphology was assigned a score of 0; mild bowel wall thickening without hyperemia was assigned a score of 1; moderate bowel wall thickening with hyperemia was assigned a score of 2; severe bowel wall thickening with rigidity and hyperemia was assigned a score of 3; and severe bowel wall thickening with rigidity, hyperemia, and adhesions was assigned a score of 4. Samples were processed and stained with hematoxylin and eosin (H&E) by the CSMC Histology Core Laboratory. Masson-Trichrome and vimentin staining were performed as described.²⁰ Histopathological scores of colons and intestine were assigned in a blinded manner by two trained animal pathologists (D.Q.S. and H.W.K.) using established methods.^{10,18} For the DSS model, histology was scored as follows: inflammation (I): 0, none; 1, mild; 2, moderate; 3, severe; extent (E): 0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural; regeneration (R): 0, complete regeneration; 1, almost complete regeneration; 2, regeneration with crypt depletion; 3, surface epithelium not intact; 4, no tissue repair; crypt damage (C): 0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, only surface epithelium intact; 4, entire crypt and epithelium lost; percentage involvement (P): 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, 76% to 100%. Total histology score is given as I + E + R + C + P. For the adoptive-transfer model, histopathological assessments of colons were scored as follows: inflammatory infiltrate in lamina propria (I): 1, 1% to 25%; 2, 26% to 66%; 3, 66% to 100%; mucin depletion (M): 0, normal; 1, up to 70% depleted; 2, over 70% depleted; epithelial atypia (E): 0, normal; 1, epithelial hyperplasia; 2, epithelial atypia; 3, epithelial atypia with nuclear changes; crypt architecture

(C): 0, normal; 1, distortion; 2, branching or atrophy; 3, complete loss; crypt abscess (A): 0, none; 1, few cells; 2, abscess; ulceration (U): 0, normal; 1, erosion; 2, ulceration; depth of involvement (D): 1, to submucosa; 2, transmural. Total colonic histology score for adoptive-transfer model is given as $I + M + E + C + A + U + D$. Histopathological scoring of small intestine in the adoptive transfer model was performed as follows: inflammation (I): 0, normal; 1, mild; 2, moderate; 3, severe; crypt damage (C): 0, none; 1, basal one-third damaged; 2, basal two-third damaged; 3, more than two-third damaged; percent involvement (P): 1, mucosa; 2, submucosa; 3, transmural; villus change (V): 0, normal; 1, distortion; 2, branching; 3, atrophy and blunting. The total intestinal score for the adoptive-transfer model is given as $I + C + P + V$. The histological score, vimentin-positive cells, and collagen thickness were calculated from observation of ≥ 10 different fields per gut region at $\times 200$ magnification from ≥ 10 mice in each group.

Expression Analysis

Total RNA was isolated and reverse-transcription polymerase chain reaction (RT-PCR) was performed as described.²¹ β -Actin, IGF-1, and TGF- β mRNA were amplified as described.^{10,20} Amount of transcript was analyzed and expressed as percentage of β -actin. Cytokine concentration was assayed by enzyme-linked immunosorbent assay (ELISA) using kits for IFN- γ , IL-17, IL-13, IL-6, and TNF α (eBioscience, San Diego, CA) per the manufacturer's protocol.

Cell Isolation, Culture, Intracellular Cytokine Expression, and Flow Cytometry

Isolation and culture of lamina propria mononuclear cells (LPMC), mesenteric lymph node (MLN), and splenic cells and their subsequent stimulation by anti-CD28 and anti-CD3 were performed as reported.¹⁰ We used the whole colon and the distal 10 cm of the ilea for LPMC isolation. Intracellular staining and antibodies used were described previously.¹⁷ Cells were acquired on a Cyan flow cytometer (Dako-Cytomation, Carpinteria, CA) and analyzed using FlowJo analysis software (Tree Star, Ashland, OR).

Human Subjects

Patients enrolled in this study were seen at the CSMC Inflammatory Bowel Disease Center and had been diagnosed with CD according to standard clinical, endoscopic, radiological, and histological findings. Written informed consent was obtained and approved by the CSMC Institutional Review Board (IRB nos. 3358 and 2673). Clinical course, laboratory values, and endoscopic findings were collected, and histology was scored by two pathologists blinded to TL1A status (D.Q.S. and M.V.). The biopsy specimens from the first available colonoscopy at CSMC after IBD diagnosis were used. Ten or more histological sections per gut

region from each patient were scored at $\times 200$ magnification.²² Assessment of TL1A levels, genotyping, and haplotype assignment had been performed.³ Patients defined as high TL1A producers were Jewish subjects with homozygous risk haplotype-B whose immune complex-stimulated TL1A at 6 hours was >0.15 ng/mL/ 1×10^6 peripheral monocytes. Patients defined as low TL1A producers were Jewish subjects with homozygous protective haplotype-A whose immune complex-stimulated TL1A at 6 hours was <0.15 ng/mL/ 1×10^6 peripheral monocytes.

Statistical Analysis

Data are presented as the mean \pm SD. Comparison between two groups was performed by a two-tailed Fisher's exact test for categorical variables and Student's *t*-test for continuous variables. Parametric and nonparametric tests were used depending on the fulfillment of the test assumptions. Comparison between three groups was done using analysis of variance test, followed by pairwise post hoc analysis with Tukey's HSD test and Behrens-Fisher test correction for the multiple comparisons. $P < 0.05$ was considered significant.

Results

Constitutive TL1A Expression Leads to Reduced Weight in the Adoptive T-Cell Transfer Model

DSS-induced and adoptive T-cell transfer models were used to determine the *in vivo* consequences of TL1A overexpression. Adaptive immunity plays an important role in chronic colitis induced by multiple cycles of DSS^{10,23} and by adoptively transferring naive CD4⁺CD45RB^{hi} T cells into immune-deficient *Rag1*^{-/-} mice.¹⁸ No significant differences in weight were observed in the DSS model (Figure 1A). In general, there was no difference in disease activity index between WT and Tg mice (data not shown). To determine that changes in the disease activity index were not due to differences in consumed DSS water between the different mice groups, DSS water consumption was assessed. We found that the quantity of consumed DSS water was similar for each of the four DSS treatment cycles among the different groups of mice (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

In the adoptive-transfer model, *Rag1*^{-/-} mice that received naive L-Tg (but not WT) T cells had significantly lower weight by week 1 that persisted up to week 6 after T-cell transfer (Figure 1B). No differences in the amount of stool blood or consistency were found (data not shown). We observed that constitutive TL1A expression led to reduced weight in the adoptive T-cell transfer model.

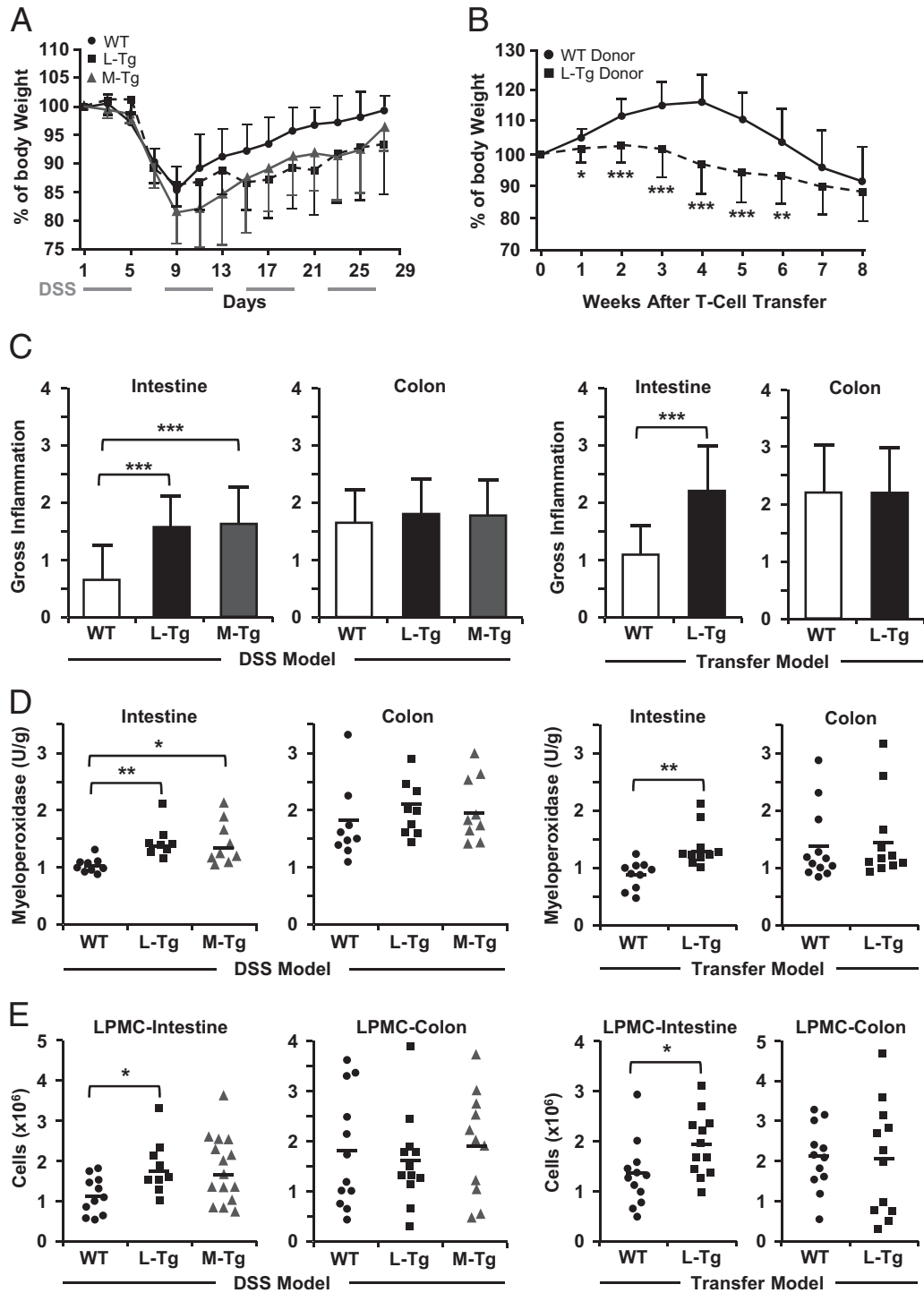


Figure 1. Intestinal and colonic disease features in WT, L-Tg, and M-Tg mice. Percentage of body weight change is shown for DSS-induced colitis ($n = 15$ for WT, M-Tg, and L-Tg) (A) and adoptive T-cell transfer model ($n = 12$ for WT donor and L-Tg donor) (B). Data are expressed as mean \pm SD. C: Gross appearance of intestine and colon were measured using a standard scoring system.¹⁸ D: Myeloperoxidase activity was measured, and data are expressed as arbitrary unit (U) per gram (g) of protein. E: Total numbers of mononuclear cells were isolated from the distal 10 cm of intestine or colon. For D, each symbol represents independent mice. Each symbol in E represents the mean of triplicates. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Increased Intestinal Inflammation in Tl1a-Tg Mice

Inspection of the gut revealed increased gross inflammation in the intestine of *Tl1a*-Tg compared to WT mice in

both chronic colitis models (Figure 1C). Another measure of gut inflammation was to determine myeloperoxidase activity.¹⁷ Compared to WT mice, increased myeloperoxidase activity was found in the ileum of *Tl1a*-Tg mice in both models of chronic colitis (Figure 1D). Additionally,

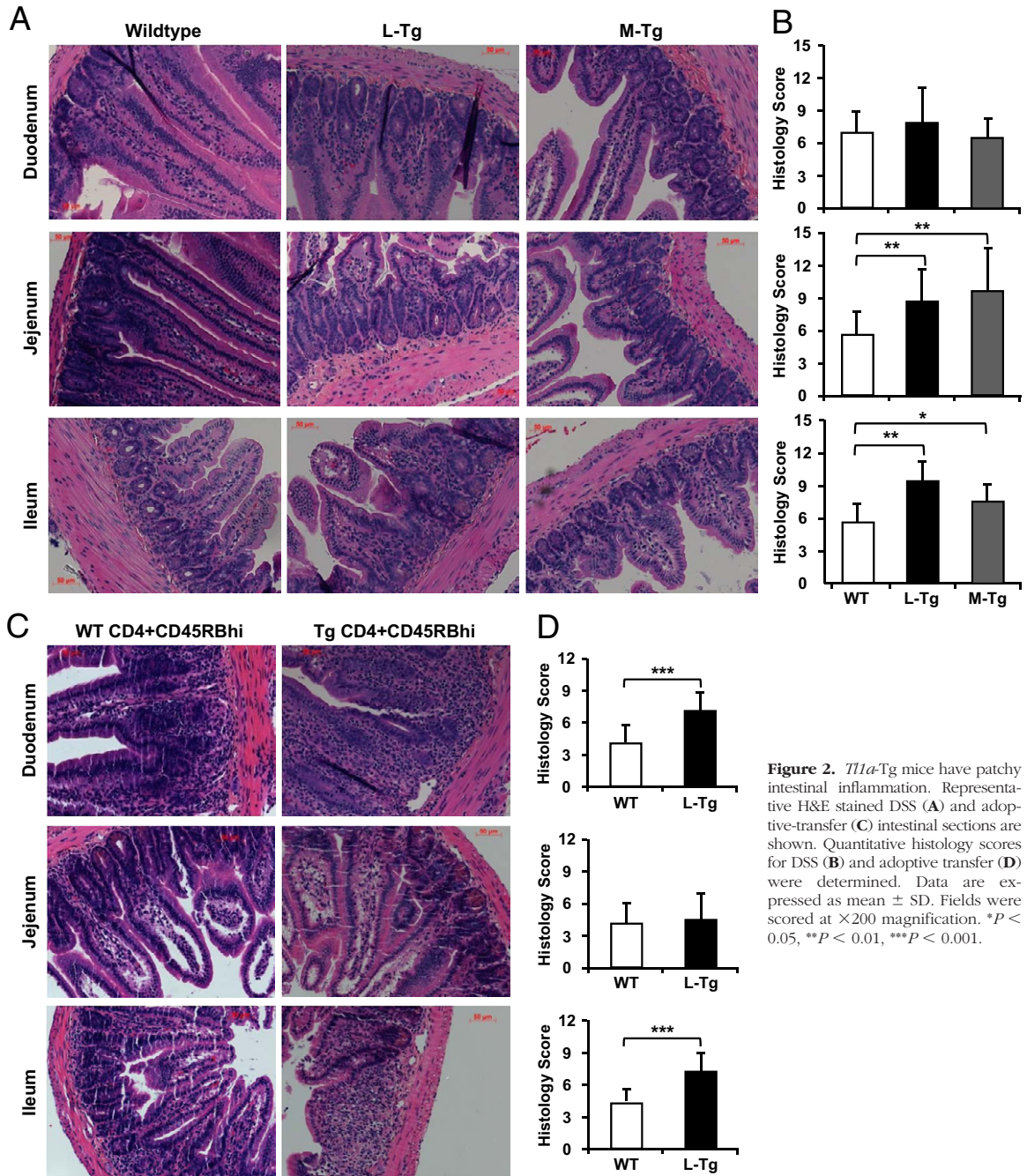


Figure 2. *T11a*-Tg mice have patchy intestinal inflammation. Representative H&E stained DSS (A) and adoptive-transfer (C) intestinal sections are shown. Quantitative histology scores for DSS (B) and adoptive transfer (D) were determined. Data are expressed as mean \pm SD. Fields were scored at $\times 200$ magnification. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

greater numbers of LPMC were isolated from the intestine of both *T11a*-Tg models than from WT mice (Figure 1E). Histological examination of the intestine revealed blunting of the villi, crypt damage, worsened inflammatory changes, and increased mononuclear cell infiltration of the lamina propria (LP), particularly in the ileum from *T11a*-Tg compared to WT mice in both the DSS (Figure 2A) and adoptive-transfer models (Figure 2C). Depending on the murine colitis model used, different intestinal areas with increased inflammatory changes were observed. For example, histological changes were reflected by a significant increase in the inflammatory score of *T11a*-Tg mice in the jejunum and ileum of the

DSS model (Figure 2B) and in the duodenum and ileum of the T-cell transfer model (Figure 2D). Taken together, these results indicate that constitutive TL1A expression in either T cells or myeloid lineages resulted in patchy intestinal inflammation.

Proximal Migration of Colonic Inflammation in *T11a*-Tg Mice

No differences were found in the overall gross inflammatory score (Figure 1C), myeloperoxidase activity (Figure 1D), or LPMC recovery (Figure 1E) in the colon between

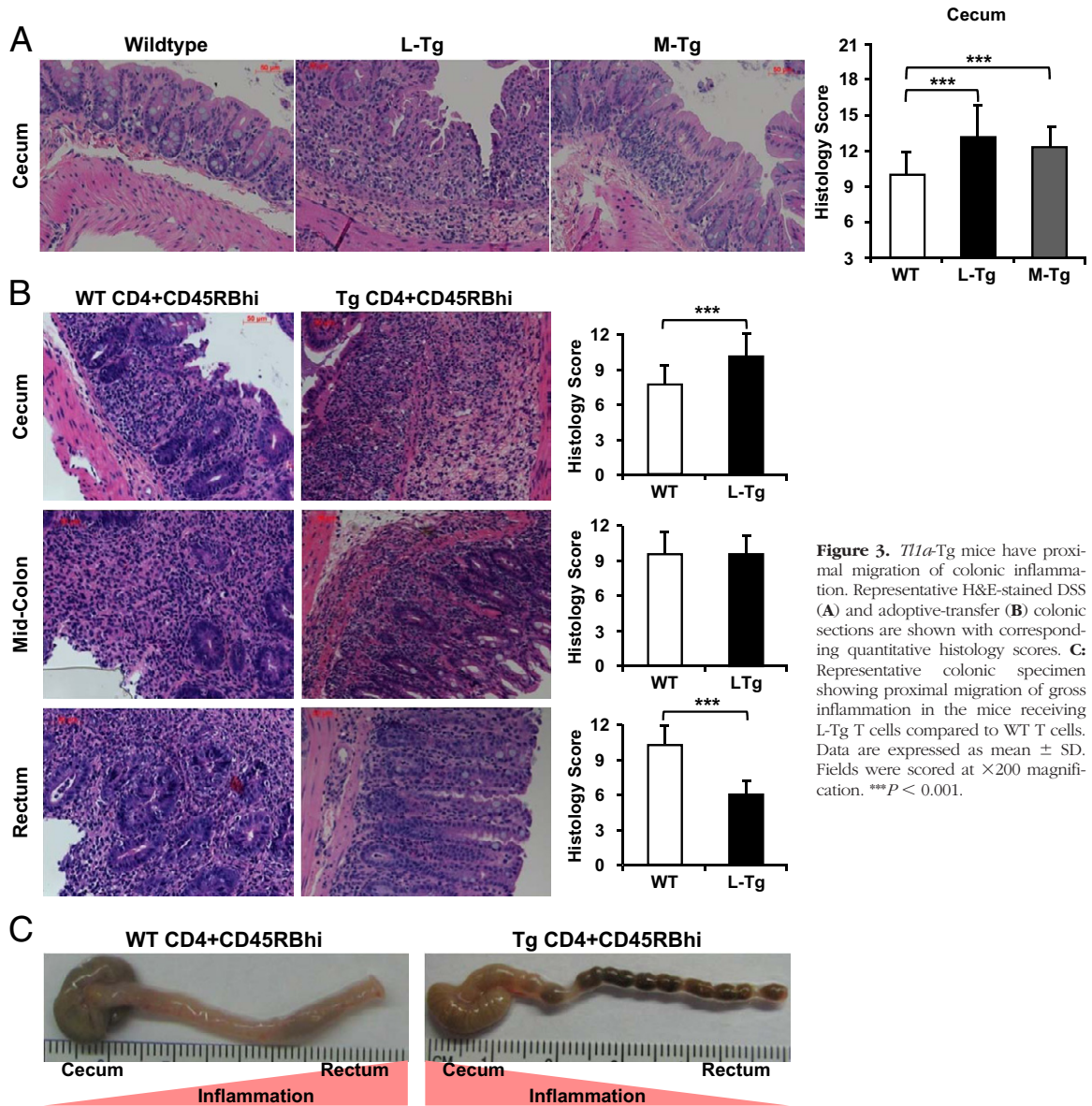


Figure 3. *T11a*-Tg mice have proximal migration of colonic inflammation. Representative H&E-stained DSS (A) and adoptive-transfer (B) colonic sections are shown with corresponding quantitative histology scores. C: Representative colonic specimen showing proximal migration of gross inflammation in the mice receiving L-Tg T cells compared to WT T cells. Data are expressed as mean ± SD. Fields were scored at ×200 magnification. ****P* < 0.001.

WT and *T11a*-Tg mice in both chronic colitis models. In the DSS-induced colitis model, enhanced inflammation was observed in the *T11a*-Tg compared to WT cecum but no other colonic regions (Figure 3A and data not shown).

In the adoptive T-cell transfer model, proximal migration of colonic inflammation was observed in T cells from *Rag1*^{-/-} mice transferred with *T11a*-Tg compared to WT T cells (Figure 3B). Compared to WT mice, there was exacerbation of cecal inflammation but relative sparing of rectal inflammation in *T11a*-Tg mice (Figure 3B). Closer inspection of colonic specimens revealed increased hyperemia, wall thickening, edema, and adhesions in the cecum with relative sparing in the rectum in the *Rag1*^{-/-} mice transferred with *T11a*-Tg compared to WT T cells (Figure 3C). These data showed constitutive TL1A expression led to proximal migration of colonic inflammation with relative rectal sparing in the adoptive T-cell transfer model.

Constitutive TL1A Expression Leads to Gut Fibrostenosis

Mice with constitutive TL1A expression developed both intestinal and colonic gross stricturing disease in both chronic colitis models. In the DSS model, gross strictures were present only in the *T11a*-Tg mice: intestine up to 3 cm in length (WT, 0 of 15 mice; L-Tg, 3 of 14 mice; M-Tg, 3 of 15 mice), colon <1 cm in length (WT, 0 of 15 mice; L-Tg, 3 of 14 mice; M-Tg, 3 of 15 mice). In the adoptive-transfer model, gross strictures were only present in *Rag1*^{-/-} mice transferred with *T11a*-Tg T cells: intestine up to 3 cm in length (WT, 0 of 12 mice; L-Tg, 4 of 12 mice), colon (WT, 0 of 12 mice; L-Tg, 3 of 12 mice). An example of fibrostenotic disease in the colon and intestine is shown in Figure 4A. At a low frequency, hydronephrosis with ureteral stricture was observed in

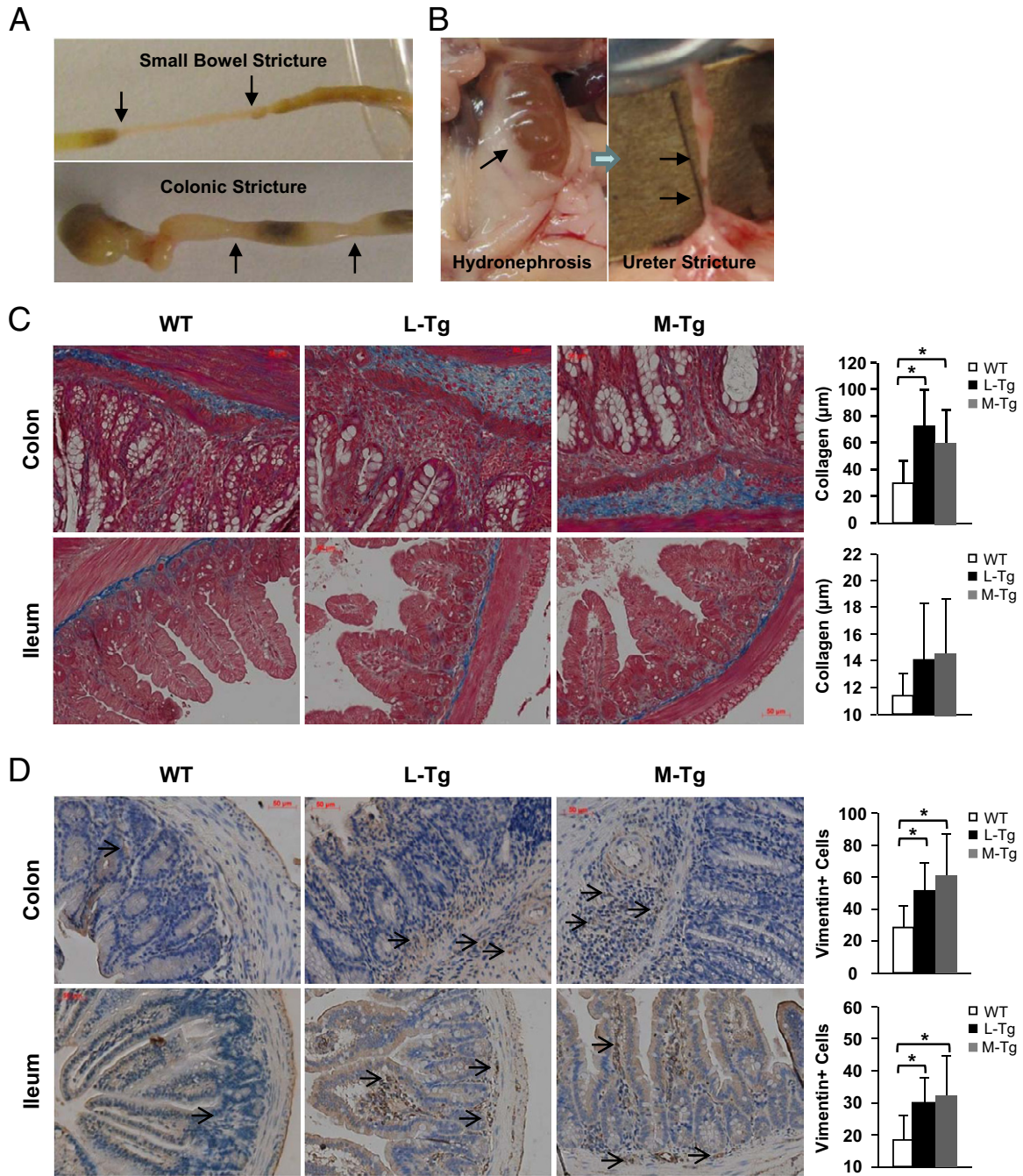


Figure 4. *T11a*-Tg mice have fibrostenotic disease. **A:** Representative pictures of intestinal and colonic stricture (arrows) are shown. **B:** Representative pictures of hydronephrosis due to ureteral stricture (arrows) are shown. **C:** Masson-Trichrome staining of collagen deposition in nonstricted tissue sections of mouse at mid-colon and distal 3 cm of ilea is shown for the DSS model. Thickness of collagen deposition (stained blue) was quantitated and represented as mean \pm SD. **D:** Vimentin stain of fibroblasts in nonstricted tissue sections were performed for the DSS model. Fibroblasts were stained brown (arrows). Vimentin-positive cells were quantitated and represented as mean \pm SD. Fields were scored at $\times 200$ magnification. * $P < 0.05$.

the DSS model from the *T11a*-Tg mice (WT, 0 of 15; L-Tg, 1 of 14; M-Tg, 1 of 15) and in the adoptive T-cell transfer model (WT, 0 of 12; L-Tg, 1 of 14). An example of hydronephrosis with ureteral stricture is shown in Figure 4B.

To determine whether there was increased collagen deposition, Masson-Trichrome stain was performed on colonic and intestinal regions without gross stricture. Increased Masson-Trichrome stain in the colon, but not in

the ilea, of *T11a*-Tg mice undergoing chronic DSS treatment was observed (Figure 4C). The increased Masson-Trichrome stain was correlated with increased thickness of colonic collagen deposition in both L-Tg and M-Tg mice when compared to WT mice (Figure 4C). Collagen is secreted by fibroblasts and can be identified by the marker vimentin.²⁰ Compared with WT controls, an increased number of vimentin-positive cells was observed in the nonstricted colon and ilea of DSS-treated *T11a*-Tg

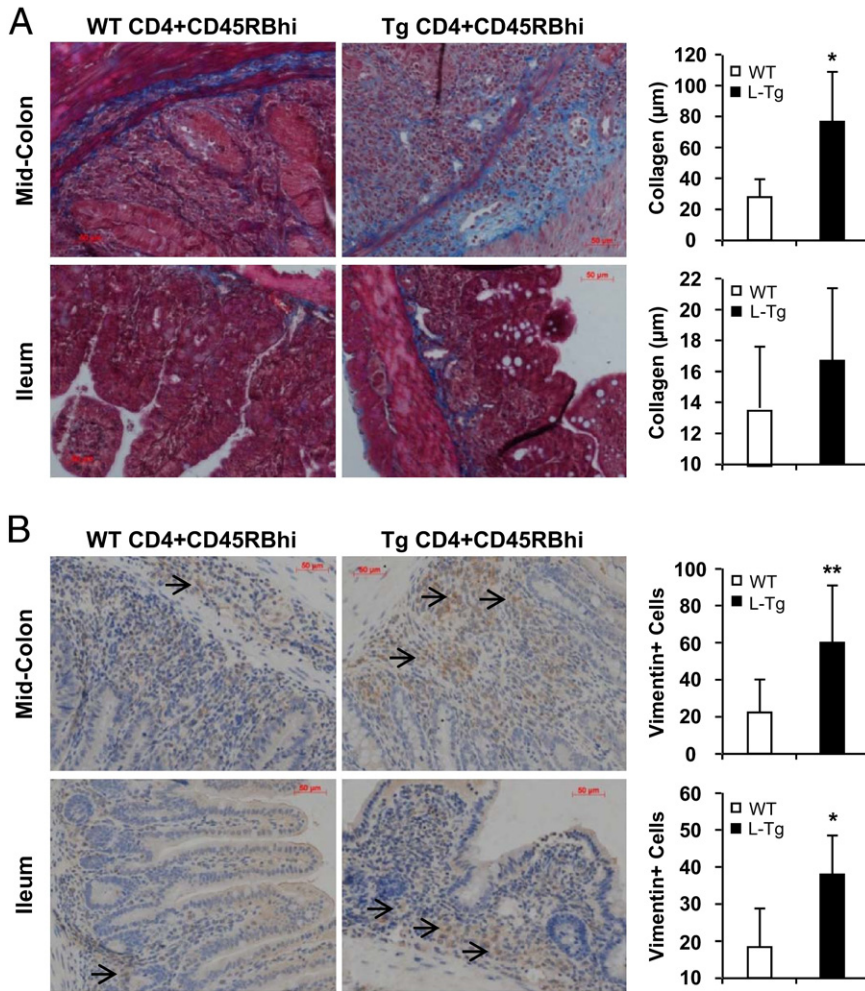


Figure 5. Adoptive transfer of L-Tg mice can lead to fibrostenotic disease. **A:** Masson-Trichrome staining of collagen deposition in nonrestricted tissue sections of mouse at mid-colon and distal 3 cm of ilea is shown for the adoptive-transfer model. Thickness of collagen deposition was quantitated and represented as mean \pm SD. **B:** Vimentin stain of fibroblasts in nonrestricted tissue sections was performed for the adoptive-transfer model. Fibroblasts were stained brown (arrows). Vimentin-positive cells were quantitated and represented as mean \pm SD. Fields were scored at $\times 200$ magnification for A and B. * $P < 0.05$, ** $P < 0.01$.

mice (Figures 4D). Similar to the DSS model, increased collagen deposition was found in the nonrestricted colon in *Rag1*^{-/-} mice transferred with *T11a* Tg T cells (Figure 5A). Collagen deposition was not different in the nonrestricted ilea (Figure 5A). Increased numbers of vimentin-positive cells were observed in both the nonrestricted ileum and colon of *Rag1*^{-/-} mice transferred with *T11a*-Tg than with WT T cells (Figure 5B).

As increased colonic mRNA expression of pro-fibrogenic factors TGF- β 1 and IGF-1 in CD patients had been reported,^{24,25} the expression of these genes was assessed. Compared to WT mice (1.1% \pm 0.34% actin), increased expression of colonic TGF- β 1 in the L-Tg (2.0% \pm 0.94%, $P = 0.02$ versus WT) and M-Tg mice (2.29% \pm 1.4% actin, $P = 0.02$ versus WT) undergoing chronic DSS treatment was found in the nonfibrotic colonic sections. The expression of TGF- β 1 in the intestine was not different between WT and *T11a*-Tg mice (data not shown). No difference in the expression of IGF-1 was detected in both chronic colitis models (data not shown). Together, these results demonstrated that constitutive TL1A expression in T cells and myeloid cells led to severe fibrostenotic disease in the murine gut, which may in part be due to elevated expression of the pro-fibrogenic factor TGF- β 1.

Accelerated T-Cell Activation and Enhanced IL-17 Production in the Chronic DSS Colitis Model

To assess the effect of TL1A expression on immune-cell composition, we performed flow cytometric analysis. Flow cytometry did not reveal differences in the frequencies of CD3⁺, CD4⁺, CD8⁺, CD11c⁺, or F4/80⁺ cells in the spleen and MLN between L-Tg, M-Tg, or WT littermate mice (data not shown). To determine the effect of constitutive TL1A expression on the activation state of antigen-presenting cells, the expression of the activation marker CD86 was compared. Flow cytometry did not reveal differences in the CD86 expression between dendritic cells (CD11c⁺) and macrophages (F4/80⁺) between *T11a*-Tg and WT mice (data not shown). Similar frequencies of T cells expressing the gut-homing markers chemokine receptor-9 and chemokine receptor-10 were found (data not shown).

To assess whether constitutive TL1A expression can enhance the co-stimulation of T cells in the DSS model, we compared the expression of CD44 on CD4⁺ and CD8⁺ cells between Tg and WT littermate controls. CD4⁺CD45RB^{low}CD25⁺ Treg cells were gated out to ex-

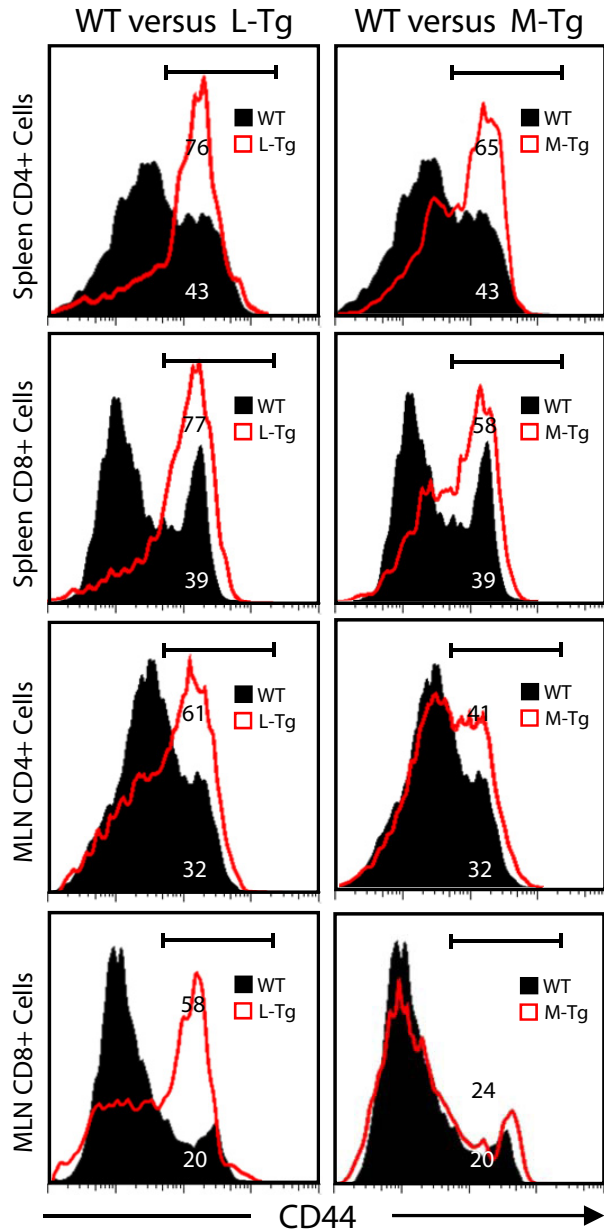


Figure 6. Sustained TL1A expression leads to increased percentage of activated T cells in DSS-induced chronic colitis. Flow cytometry plot of splenocytes and MLN cells showing expression of activation markers CD44. CD4⁺ and CD8⁺ cells were gated as indicated. Data shown are representative of at least five independent experiments.

amine the expression of activation markers on conventional T cells. Compared to WT mice, a higher percentage of *T11a*-Tg CD4⁺ and CD8⁺ cells expressed the activation marker CD44, particularly in the L-Tg mice (Figure 6).

To assess the molecular consequences of increased T-cell activation in the *T11a*-Tg mice, the expression of IFN- γ , interleukin (IL)-13, and IL-17 was measured. Figure 7A shows increased frequency of CD4⁺IL-17⁺ T cells in *T11a*-Tg mice compared to WT mice that reached significance in the frequency of CD4⁺IL-17⁺ in the MLN (WT 0.6 \pm 0.2 versus L-Tg 6.0 \pm 2.8, $P = 0.03$; WT 0.6 \pm 0.2 versus M-Tg 4.0 \pm 1.9, $P = 0.04$). No difference in the proportion of CD4⁺IL-13⁺ and CD4⁺IFN- γ ⁺ cells was

found. Similar to what was shown with intracellular staining; higher IL-17 production in the MLN for both *T11a*-Tg mice was found (Figure 7B). No differences in the production of IFN- γ , IL-13, TNF α , or IL-6 were observed (Figure 7B and data not shown).

Constitutive TL1A Expression Leads to Enhanced Th-1 but Reduced Th-17 Immune-Responses in the Adoptive T-Cell Transfer Model

In the adoptive T-cell transfer model, no differences in the frequencies of CD3⁺, CD4⁺, chemokine receptor-9 and -10, activation markers (CD44 and CD86), CD11c⁺, or F4/80⁺ cells in the spleen, and MLN between *Rag1*^{-/-} mice transferred with WT or *T11a*-Tg T cells were observed (data not shown). Intracellular cytokine staining showed that the frequency of CD4⁺IFN- γ ⁺ was significantly elevated in the spleen (WT 7.7 \pm 0.9 versus L-Tg 11.1 \pm 1.5, $P = 0.03$) and MLN (WT 9.5 \pm 0.8 versus L-Tg 13.1 \pm 2.0, $P = 0.04$) of *Rag1*^{-/-} mice transferred with T cells expressing TL1A compared to WT T cells (Figure 8A). There were very low numbers of CD4⁺IL-17⁺ T cells in *Rag1*^{-/-} mice transferred with *T11a*-Tg T cells in the spleen (WT 11.5 \pm 3.4 versus L-Tg 4.4 \pm 2.1, $P = 0.04$) and MLN (WT 18.3 \pm 2.2 versus L-Tg 1.7 \pm 1.2, $P < 0.01$) (Figure 8A). No differences in the frequency of CD4⁺IL13⁺ cells were found (Figure 8A).

Consistent with the results from intracellular staining, higher IFN- γ production was found in the spleen, MLN, and LPMC in *Rag1*^{-/-} mice transferred with *T11a*-Tg T cells compared to WT T cells (Figure 8B). Significantly reduced IL-17 production was observed in all of the tissues tested (Figure 8B). No differences in the production of IL-13, TNF α , and IL-6 were observed (Figure 8B and data not shown). These data indicated that constitutive expression of TL1A in naive CD4⁺CD45RB^{hi} T cells may affect the proliferation, differentiation, or maintenance of Th-1 and -17 cells in the peripheral immune compartments and inflamed tissues.

Proximal Migration of Colonic Inflammation with Relative Rectal Sparing and Intestinal Fibrostenosis in High TL1A Expressing CD Patients

Given the findings in mice, we examined whether humans with elevated TL1A expression had similar proximal migration of colonic inflammation with relative rectal sparing. A blinded retrospective analysis of CD patients with known TL1A expression in peripheral monocytes was performed.³ In our previous study, TL1A level of 0.15 ng/mL/1 $\times 10^6$ peripheral monocytes at 6 hours post immune complex stimulation distinguished over 90% of Jewish CD patients with risk *TL1A* haplotype-B and protective haplotype-A.³ Therefore, high TL1A producers were defined as subjects with homozygous risk haplotype-B whose immune complex-stimulated TL1A at 6 hours was ≥ 0.15 ng/mL/1 $\times 10^6$ peripheral monocytes,

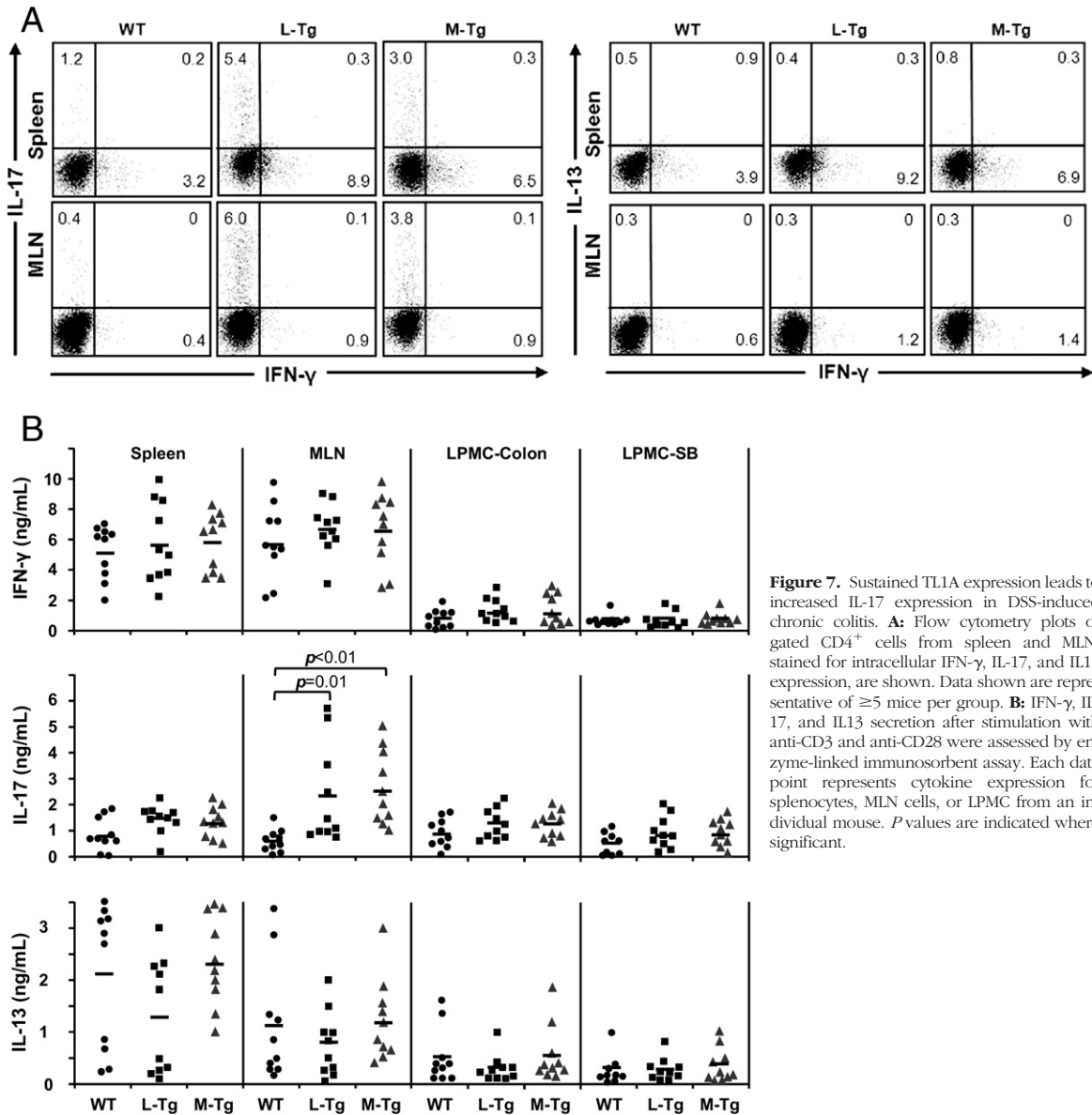


Figure 7. Sustained TL1A expression leads to increased IL-17 expression in DSS-induced chronic colitis. **A:** Flow cytometry plots of gated CD4⁺ cells from spleen and MLN, stained for intracellular IFN- γ , IL-17, and IL13 expression, are shown. Data shown are representative of ≥ 5 mice per group. **B:** IFN- γ , IL-17, and IL13 secretion after stimulation with anti-CD3 β and anti-CD28 were assessed by enzyme-linked immunosorbent assay. Each data point represents cytokine expression for splenocytes, MLN cells, or LPMC from an individual mouse. *P* values are indicated where significant.

and low TL1A producers had homozygous protective haplotype-A whose immune complex-stimulated TL1A at 6 hours was ≤ 0.15 ng/mL/ 1×10^6 peripheral monocytes. The patients' demographics, clinical features, and medications within 2 months of colonoscopy are provided in Table 1. Significant increases in intestinal strictures and in scores from the Harvey-Bradshaw Index were found in the high-TL1A compared to the low-TL1A group (Table 1).

To assess whether TL1A expression level is associated with a specific mucosal pattern of inflammation, H&E-stained histological sections from ileum, cecum, sigmoid, and rectum were examined. Compared to the low-TL1A group, the high-TL1A group had more severe ileocecal inflammation with relative rectosigmoid sparing of inflammation (Figure 9). Together, these data suggest that CD patients who express elevated TL1A levels develop worsened inflammation in the ileoco-

lonic region and proximal migration of colonic inflammation with relative sparing of inflammation in the sigmoid and rectum.

Discussion

This study shows that constitutive TL1A expression resulted in severe patchy intestinal inflammation with proximal migration of colonic inflammation under colitogenic conditions. Furthermore, *Tl1a*-Tg mice spontaneously developed ileitis, but not colitis, by 10 months of age.¹⁵⁻¹⁷ The severity of ileitis was more dramatic in these induced chronic colitis models. As colitis is the predominant gut pathology seen in both the DSS and adoptive-transfer models, the ileitis reported in this study was likely driven by constitutive TL1A expression. Depending on the model, TL1A expression led to

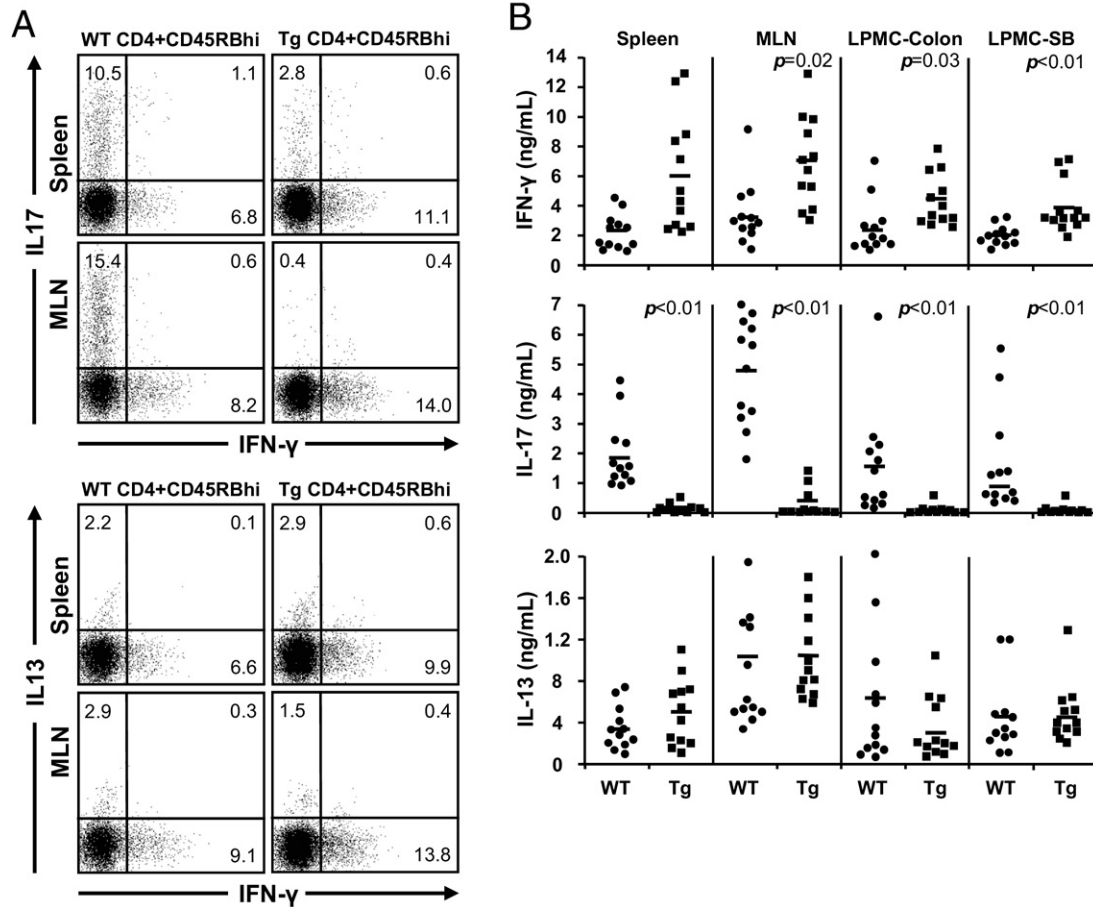


Figure 8. Sustained TL1A expression leads to increased IFN- γ and reduced IL-17 expression in the adoptive-transfer model. **A:** Flow cytometry plots of gated CD4⁺ cells from spleen and MLN, stained for intracellular IFN- γ , IL-17, and IL13 expression, are shown. Data shown are representative of ≥ 5 mice per group. **B:** IFN- γ , IL-17, and IL13 secretion after stimulation with anti-CD3 and anti-CD28 were assessed by enzyme-linked immunosorbent assay. Each data point represents cytokine expression for splenocytes, MLN cells, or LPMC of an individual mouse. *P* values are indicated where significant.

either additional inflammation in the jejunum (DSS) or duodenum (adoptive-transfer). Constitutive TL1A expression also altered the colitis pattern from the more distal rectosigmoid inflammation to the more proximal cecal inflammation. These inflammatory changes were likely caused by TL1A and not the cell type that expresses TL1A as both L-Tg and M-Tg mice exhibited a similar pattern of inflammation. Relative sparing of rectal inflammation was seen in the adoptive-transfer model using *T11a*-Tg T cells. *TNFSF15* is a severity gene, which when overexpressed results in a change in location to the proximal parts of the colon and small intestine, and away from the rectum.

Prior studies reported that TL1A is associated with expansion of T-helper subsets and can synergize with IL-12 and -18 to enhance Th-1 response and IFN- γ production.^{6,7,26,27} Other investigations found that mice lacking TL1A display reduced experimental encephalitis disease activity that is associated with decreased Th-17 cell differentiation and proliferation.⁹ Consistently, in DSS-induced models of chronic colitis, TL1A enhances Th-1 and Th-17 effector functions by up-regulating IFN- γ and IL-17 production, respectively, in CD4⁺ T cells under Th-1/Th-17 polarizing

conditions.¹⁰ TL1A had also been found to be involved in Th-2-mediated ova-induced lung disease and in spontaneous ileitis.^{8,15,16} These reports indicated that the TL1A/DR3 signaling pathway might have a universal role in regulating T-helper effector outcomes.

In line with our previous report,¹⁰ increased IL-17 production was found in DSS-induced chronic colitis with constitutive TL1A expression. In the adoptive-transfer model, TL1A expression led to a Th-1-dominant immune response with almost a complete absence of IL-17 production. The differences in the T-helper response may be based on the differentiation state of the CD4⁺ T cells. In the DSS-induced chronic colitis model, TL1A can enhance the effector characteristics of committed Th-17 cells. In the adoptive-transfer model, TL1A/DR3 signaling may inhibit Th-17 development in newly challenged naive T cells. Differential responsiveness of naive and committed T cells to TL1A/DR3 signaling had also been recently proposed.¹²

Another explanation for the difference in the TL1A-dependent T-helper immune response between the DSS and adoptive T-cell transfer models may be different gut microflora. We bred WT and *T11a*-Tg litter-

Table 1. Summary of Clinical and Laboratory Data

	High TL1A	Low TL1A	P value
Sex (M/F)	4/4	6/4	NS
SB stricture	62.5% (5/8)	10% (1/10)	0.04
Colon stricture	12.5% (1/8)	20% (2/10)	NS
Biologic use	25% (2/8)	70% (7/10)	NS
Failed 1 biologic*	62.8% (5/8)	5/10 (50%)	NS
Failed ≥2 biologics*	25% (2/8)	0%	NS
Immunomodulator† use	27.5% (3/8)	60% (6/10)	NS
Immunomodulator† failure	75% (6/8)	50% (5/10)	NS
5-ASA‡ failure	87.5% (7/8)	80% (8/10)	NS
Enema use	0%	0%	NS
Ureteral stricture	25% (2/8)	0%	NS
Previous surgery	87.5% (7/8)	70% (7/10)	NS
TL1A (ng/mL)	0.29 ± 0.12	0.08 ± 0.04	0.01
Harvey-Bradshaw Index	9 ± 3.45	2.61 ± 3.43	<0.01
Time to surgery (years)	11 ± 10.05	8.75 ± 6.8	NS
WBC (×100/μL)	8.40 ± 4.34	8.10 ± 3.31	NS
ESR (mm/hour)	20.64 ± 15.31	14.36 ± 7.20	NS
CRP (mg/dL)	1.17 ± 1.59	1.76 ± 3.31	NS

Data are expressed as percentages with *n*/total or as the mean ± SD as indicated.

*Biologics include infliximab, adalimumab, certolizumab, thalidomide, and natalizumab.

†Immunomodulators include azathioprine, 6-mercaptopurine, methotrexate, and tacrolimus.

‡5-ASAs include mesalamine and sulfasalazine.

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; F, female; M, male; NS, not significant; SB, small bowel; WBC, white blood cell count.

mates in our facility for the DSS-induced chronic colitis experiments. For the adoptive-transfer model, we purchased *Rag1*^{-/-} recipient mice from Jackson Laboratory (Bar Harbor, ME). Previous studies had demon-

strated that genetically identical inbred mice raised in different animal facilities had different immune composition due to the different gut microflora.^{28–30} The potential immunomodulatory roles of gut microbiota–host

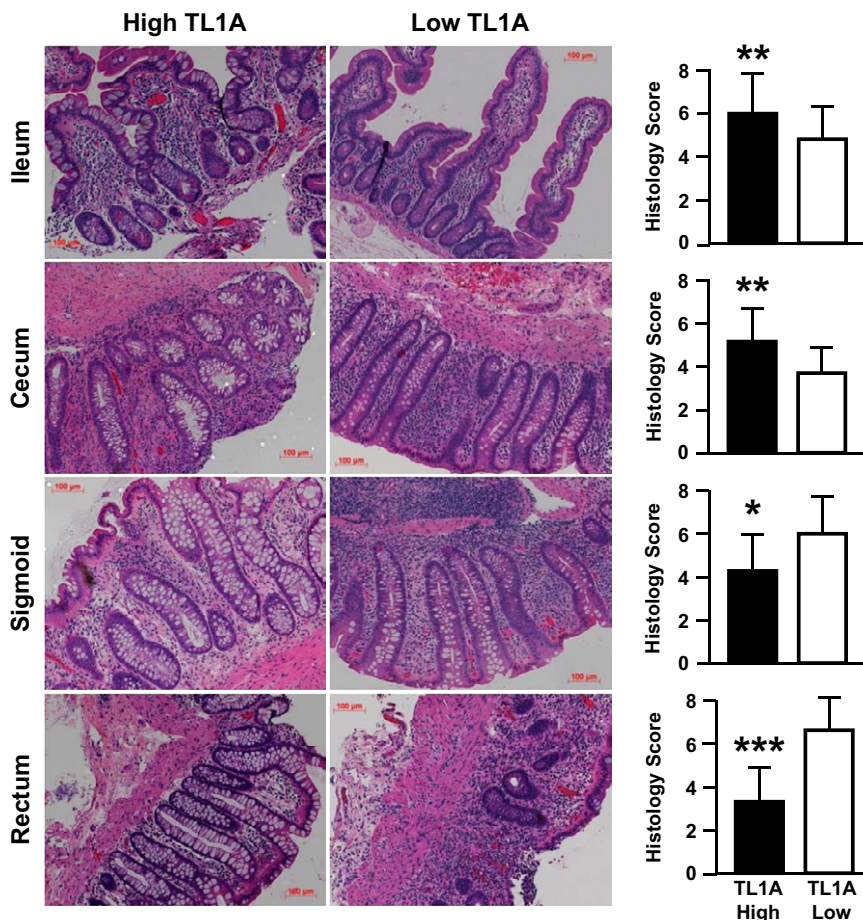


Figure 9. CD patients with elevated TL1A expression have increased ileocecal inflammation but reduced rectosigmoid inflammation. Representative H&E-stained intestinal and colonic sections are shown, and corresponding histology scores were determined. Data are expressed as mean ± SD. Sections were scored at ×200 magnification by two pathologists blinded to the TL1A haplotype and its expression level. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

interaction between mice may have contributed to the different TL1A-mediated T-helper immune response.

Compared to WT mice, increased collagen deposition was observed in older *T11a*-Tg mice.¹⁷ Notably, the severity of fibrosis was worse in the induced chronic colitis models with constitutive TL1A expression where gross (not just histological) colonic and intestinal strictures were observed. This may reflect the fact that the induced chronic colitis models led to an accelerated T-helper effector function in which the effector phase of the disease is reached earlier. In addition, TL1A may be expressed by an independent pro-fibrogenic gene to initiate the process of fibrosis since we previously found that TL1A expression resulted in increased colonic collagen deposition in the absence of detectable histological inflammation.¹⁷ Furthermore, in the current study, visible colonic strictures were observed in the mid-colon of only the *T11a*-Tg mice despite a similar degree of inflammation as compared to WT mice (Figures 3 and 4). The pro-fibrotic effects of TL1A may be mediated by TGF- β 1 as its expression was increased in the nonstrictured colon of Tg compared to WT mice (Figure 5C). However, other factors are likely involved and need to be explored further. To our knowledge, these *T11a*-Tg animal models are the only *in vivo* model in which overexpression of a single IBD-associated gene lead to gross intestinal and colonic fibrostenotic disease that is characteristic of some forms of CD.

Compared to CD patients with low TL1A expression, higher TL1A-producing CD patients had increased inflammation in the ileum and cecum with reduced inflammation in the sigmoid and rectum (Figure 9). None of the CD patients in our study cohort used rectally administered IBD therapy; therefore, the reduction in rectosigmoid inflammation may be due to elevated TL1A expression. A significantly higher rate of intestinal strictures was found in CD patients with higher TL1A levels (Table 1), suggesting that TL1A may induce intestinal strictures in humans. This is consistent with previous reports showing that *TNFSF15* haplotype-B, which is associated with increased TL1A levels, is also characterized by the increased need for surgery due to fibrostenotic CD disease.^{3,4} One limitation of the retrospective analysis is the small sample size, thus our findings need to be confirmed in an independent prospective study.

Extraintestinal manifestations of CD can include arthropathy, erythema nodosum, and more rarely, hydronephrosis due to strictures along the genitourinary tract.^{1,31,32} The presence of IBD-like skin lesions and arthropathy in older *T11a*-Tg mice were reported.¹⁷ In this study, hydronephrosis due to ureteral strictures was only observed in *T11a*-Tg mice during the development of chronic colitis (7%, 3 of 43). Ureteral involvement has been reported to occur in 3% to 6% of CD cases.³² Interestingly, a higher frequency of ureteral stricturing was found in our small cohort of CD patients expressing higher TL1A levels (25%, 2 of 8, Table 1), suggesting that TL1A may also cause ureteral fibrosis.

In summary, TL1A modulates the severity and location of inflammation in the intestine and colon under colito-

genic conditions. Sustained TL1A expression also induces stricturing disease in the gut that is caused by increased collagen deposition and number of fibroblasts. Results from these *T11a*-Tg experimental models coupled with findings in CD patients suggest that *TNFSF15* is a severity gene and that the cytokine TL1A, when overexpressed, plays a central role in generating one phenotypic form of complicated CD.

Acknowledgments

We thank Loren Karp for critical reading of the manuscript, Patricia Lin and Gislaine Martins for assistance with flow cytometry, Jeremy Chen for assistance in making histology figures, and Hanlin Wang for assistance with obtaining human histology.

References

1. Peyrin-Biroulet L, Loftus EV Jr., Colombel JF, Sandborn WJ: The natural history of adult Crohn's disease in population-based cohorts. *Am J Gastroenterol* 2010, 105:289–297
2. Shih DQ, Targan SR: Insights into IBD pathogenesis. *Curr Gastroenterol Rep* 2009, 11:473–480
3. Michelsen KS, Thomas LS, Taylor KD, Yu QT, Mei L, Landers CJ, Derkowski C, McGovern DP, Rotter JI, Targan SR: IBD-associated TL1A gene (*TNFSF15*) haplotypes determine increased expression of TL1A protein. *PLoS One* 2009, 4:e4719
4. Picornell Y, Mei L, Taylor K, Yang H, Targan SR, Rotter JI: *TNFSF15* is an ethnic-specific IBD gene. *Inflamm Bowel Dis* 2007, 13:1333–1338
5. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007, 447:661–678
6. Prehn JL, Mehdizadeh S, Landers CJ, Luo X, Cha SC, Wei P, Targan SR: Potential role for TL1A, the new TNF-family member and potent costimulator of IFN-gamma, in mucosal inflammation. *Clin Immunol* 2004, 112:66–77
7. Papadakis KA, Prehn JL, Landers C, Han Q, Luo X, Cha SC, Wei P, Targan SR: TL1A synergizes with IL-12 and IL-18 to enhance IFN-gamma production in human T cells and NK cells. *J Immunol* 2004, 172:7002–7007
8. Fang L, Adkins B, Deyev V, Podack ER: Essential role of TNF receptor superfamily 25 (*TNFRSF25*) in the development of allergic lung inflammation. *J Exp Med* 2008, 205:1037–1048
9. Pappu BP, Borodovsky A, Zheng TS, Yang X, Wu P, Dong X, Weng S, Browning B, Scott ML, Ma L, Su L, Tian Q, Schneider P, Flavell RA, Dong C, Burkly LC: TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated autoimmune disease. *J Exp Med* 2008, 205:1049–1062
10. Takedatsu H, Michelsen KS, Wei B, Landers CJ, Thomas LS, Dhall D, Braun J, Targan SR: TL1A (*TNFSF15*) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation. *Gastroenterology* 2008, 135:552–567
11. Meylan F, Davidson TS, Kahle E, Kinder M, Acharya K, Jankovic D, Bundoc V, Hodges M, Shevach EM, Keane-Myers A, Wang EC, Siegel RM: The TNF-family receptor DR3 is essential for diverse T cell-mediated inflammatory diseases. *Immunity* 2008, 29:79–89
12. Jones GW, Stumhofer JS, Foster T, Twohig JP, Hertzog P, Topley N, Williams AS, Hunter CA, Jenkins BJ, Wang EC, Jones SA: Naive and activated T cells display differential responsiveness to TL1A that affects Th17 generation, maintenance, and proliferation. *FASEB J* 2011, 25:409–419
13. Schreiber TH, Wolf D, Podack ER: The role of *TNFRSF25*: *TNFSF15* in disease. and health? *Adv Exp Med Biol* 2011, 691:289–298
14. Shih DQ, Michelsen KS, Barrett RJ, Biener-Ramanujan E, Gonsky R, Zhang X, Targan SR: Insights into TL1A and IBD pathogenesis. *Adv Exp Med Biol* 2011, 691:279–288
15. Meylan F, Song YJ, Fuss I, Villarreal S, Kahle E, Malm IJ, Acharya K, Ramos HL, Lo L, Mentink-Kane MM, Wynn TA, Migone TS, Strober W,

- Siegel RM: The TNF-family cytokine TL1A drives IL-13-dependent small intestinal inflammation. *Mucosal Immunol* 2011, 4:172–185
16. Taraban VY, Slebiada TJ, Willoughby JE, Buchan SL, James S, Sheth B, Smyth NR, Thomas GJ, Wang EC, Al-Shamkhani A: Sustained TL1A expression modulates effector and regulatory T-cell responses and drives intestinal goblet cell hyperplasia. *Mucosal Immunol* 2011, 4:186–196
 17. Shih DQ, Barrett R, Zhang X, Yeager N, Koon HW, Phaosawasdi P, Song Y, Ko B, Wong MH, Michelsen KS, Martins G, Pothoulakis C, Targan SR: Constitutive TL1A (TNFSF15) expression on lymphoid or myeloid cells leads to mild intestinal inflammation and fibrosis. *PLoS One* 2011, 6:e16090
 18. Ostanin DV, Pavlick KP, Bharwani S, D'Souza D, Furr KL, Brown CM, Grisham MB: T cell-induced inflammation of the small and large intestine in immunodeficient mice. *Am J Physiol Gastrointest Liver Physiol* 2006, 290:G109–G119
 19. Rachmilewitz D, Karmeli F, Shteingart S, Lee J, Takabayashi K, Raz E: Immunostimulatory oligonucleotides inhibit colonic proinflammatory cytokine production in ulcerative colitis. *Inflamm Bowel Dis* 2006, 12:339–345
 20. Koon HW, Shih D, Karagiannides I, Zhao D, Fazelbhoj Z, Hing T, Xu H, Lu B, Gerard N, Pothoulakis C: Substance P modulates chronic inflammation-induced colonic fibrosis. *Am J Pathol* 2010, 177:2300–2309
 21. Shih DQ, Kwan LY, Chavez V, Cohavy O, Gonsky R, Chang EY, Chang C, Elson CO, Targan SR: Microbial induction of inflammatory bowel disease associated gene TL1A (TNFSF15) in antigen presenting cells. *Eur J Immunol* 2009, 39:3239–3250
 22. D'Haens GR, Geboes K, Peeters M, Baert F, Penninckx F, Rutgeerts P: Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. *Gastroenterology* 1998, 114:262–267
 23. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO: Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 1994, 107:1643–1652
 24. Lawrance IC, Maxwell L, Doe W: Inflammation location, but not type, determines the increase in TGF-beta1 and IGF-1 expression and collagen deposition in IBD intestine. *Inflamm Bowel Dis* 2001, 7:16–26
 25. Zimmermann EM, Li L, Hou YT, Mohapatra NK, Pucilowska JB: Insulin-like growth factor I and insulin-like growth factor binding protein 5 in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 2001, 280:G1022–G1029
 26. Bamias G, Martin C 3rd, Marini M, Hoang S, Mishina M, Ross WG, Sachedina MA, Friel CM, Mize J, Bickston SJ, Pizarro TT, Wei P, Cominelli F: Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. *J Immunol* 2003, 171:4868–4874
 27. Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen SF, Zhou JX, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gantz R, Feng P, Moore PA, Ruben SM, Wei P: TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* 2002, 16:479–492
 28. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR: Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 2009, 139:485–498
 29. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, Littman DR: Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 2008, 4:337–349
 30. Wu HJ, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, Littman DR, Benoist C, Mathis D: Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 2010, 32:815–827
 31. Kruglik GD, Neiman HL, Sparberg M, Nudelman E, Mintzer RA, Rogers LF: Urological complications of regional enteritis. *Gastrointest Radiol* 1977, 1:375–378
 32. Ruffolo C, Angriman I, Scarpa M, Polese L, Barollo M, Bertin M, Pagano D, D'Amico DF: Minimally invasive management of Crohn's disease complicated by ureteral stenosis. *Surg Laparosc Endosc Percutan Tech* 2004, 14:292–294