

BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Inhibition of Plasmin Protects Against Colitis in Mice by Suppressing Matrix Metalloproteinase 9–Mediated Cytokine Release From Myeloid Cells



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BACKGROUND & AIMS: Activated proteases such as plasmin and matrix metalloproteinases (MMPs) are activated in intestinal tissues of patients with active inflammatory bowel diseases. We investigated the effect of plasmin on the progression of acute colitis. **METHODS:** Colitis was induced in *Mmp9*^{-/-}, *Plg*^{-/-}, and C57BL/6 (control) mice by the administration of dextran sulfate sodium, trinitrobenzene sulfonic acid, or CD40 antibody. Plasmin was inhibited in control mice by intraperitoneal injection of YO-2, which blocks its active site. Mucosal and blood samples were collected and analyzed by reverse-transcription polymerase chain reaction and immunohistochemical analyses, as well as for mucosal inflammation and levels of cytokines and chemokines. **RESULTS:** Circulating levels of plasmin were increased in mice with colitis, compared with controls. Colitis did not develop in control mice injected with YO-2 or in *Plg*^{-/-} mice. Colons from these mice had reduced infiltration of Gr1+ neutrophils and F4/80+ macrophages, and reduced levels of inflammatory cytokines and chemokines. Colonic inflammation and colitis induction required activation of endogenous MMP9. After colitis induction, mice given YO-2, *Plg*^{-/-} mice, and *Mmp9*^{-/-} mice had reduced serum levels of tumor necrosis factor and C-X-C motif chemokine ligand 5, compared with control mice. **CONCLUSIONS:** In mice, plasmin induces a feedback mechanism in which activation of the fibrinolytic system promotes the development of colitis via activation of MMP9 or proteolytic enzymes. The proteolytic environment stimulates the influx of myeloid cells into the colonic epithelium and the production of tumor necrosis factor and C-X-C motif chemokine ligand 5. In turn, myeloid CD11b+ cells release the urokinase plasminogen activator, which accelerates plasmin production. Disruption of the plasmin-induced chronic inflammatory circuit therefore might be a strategy for colitis treatment.

immunopathologic process leading to chronic inflammation.¹ Treatment of IBD generally relieves symptoms, but is not curative. Activation of proteases such as matrix metalloproteinases (MMPs), or serine proteases, such as plasmin, can break down the intestinal-epithelial barrier because of their potential to degrade components of the extracellular matrix, resulting in the invasion of inflammatory cells.²

MMP9, expressed by epithelial cells, plays an important role in the development of colitis by modulating cell-matrix interaction and wound healing.³ MMP inhibitor treatment is effective for colitis, but patients have described side effects including severe lethargy and chills. Therefore, the therapeutic effects of more selective inhibitors of disease-associated MMPs currently are under investigation.⁴ Another way of controlling the activation of MMPs is through the serine protease plasmin,^{5–7} which has been shown to be important for the release of cytokines/chemokines such as Kit ligand, monocyte chemoattractant protein-1, C-X-C motif chemokine ligand 5 (CXCL5), and basic fibroblastic growth factor.^{7–11} It is conceivable that the production of proinflammatory cytokines such as tumor necrosis factor α (TNF- α) also is regulated via plasmin-mediated MMP activation. TNF- α is induced rapidly in the intestinal mucosa during the initial activation of immune cells, and this induction is linked to disease progression during IBD.¹²

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Abbreviations used in this paper: Ab, antibody; CXCL5, C-X-C motif chemokine ligand 5; DAJ, disease activity index; DSS, dextran sulfate sodium; FDP, fibrin degradation product; IBD, inflammatory bowel disease; IL, interleukin; MMP, matrix metalloproteinase; PA, plasminogen activator; PAP, plasmin-antiplasmin complex; Plg, plasminogen; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; uPA/Plau, urokinase-type plasminogen activator; YO-2, trans-4-aminomethylcyclohexanecarbonyl-Tyr(O-Pic)-octylamide.

Keywords: IBD; Mouse Model; Plasminogen; UC.

Crohn's disease and ulcerative colitis, the major forms of inflammatory bowel disease (IBD) in human beings, result from the interaction of genetic and environmental factors that ultimately promote an

Plasmin, a key enzyme of the fibrinolytic cascade, can degrade the fibrin clot. It is generated by conversion from its precursor, plasminogen (Plg), by the plasminogen activators (PAs) tissue-type PA (Plat), and urokinase-type PA (uPA/Plau). Early clinical studies showed that circulating monocytes derived from IBD patients showed increased secretion of PA,¹³ and that disease activity depended on up-regulation of uPA in the active stage of the disease.¹⁴

In the present study, we examined the role of the fibrinolytic system in patients with IBD. We show that genetic and pharmacologic plasmin inhibition prevents the progression of IBD in experimental models of colitis, and ameliorates the disease in part by suppressing the MMP9-dependent influx of inflammatory cells and production of inflammatory cytokines.

Materials and Methods

Animal Studies

Mmp9^{+/+} and *Mmp9*^{-/-} mice and *Plg*^{+/+} and *Plg*^{-/-} mice each were used after 10 back-crosses onto a C57BL/6 background. C57BL/6 recombinase-activating gene 2 (*Rag2*^{-/-}) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). Animal studies were approved by the Animal Review Board of Juntendo University (Tokyo, Japan).

Induction of Colitis

Experimental dextran sulfate sodium (DSS) (ICN Biomedical molecular weight, 36,000–50,000 daltons; ICN Biomedicals Inc, Ohio, GA) colitis was induced by administering 2% DSS via drinking water on days 0–7. Trinitrobenzene sulfonic acid (TNBS)-induced colitis was induced by colonic injection of 100 μ L

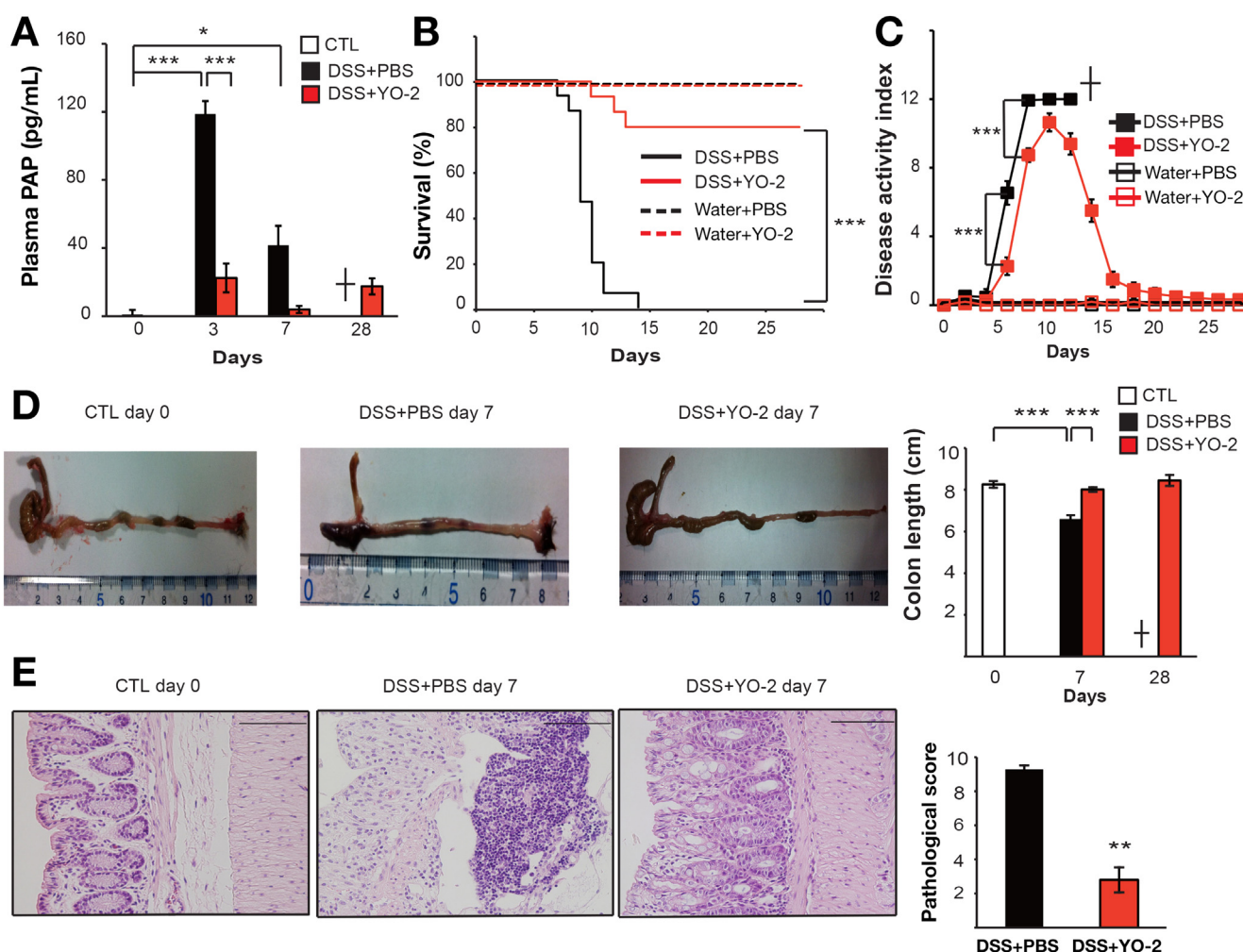


Figure 1. Plasmin inhibition prevents DSS-induced colitis progression. Colitis was induced by DSS and mice were injected with or without YO-2. (A) Plasma derived from mice treated with or without YO-2 was analyzed for PAP as a measure of active plasmin by enzyme-linked immunosorbent assay. $n = 3$ /group. (B) Percentage survival and (C) DAI were determined at the indicated time points in the following treatment groups: 2% DSS + phosphate-buffered saline (PBS), $n = 15$; 2% DSS + YO-2, $n = 15$; water + PBS and water + YO-2, $n = 5$. (D) Colon lengths were measured at the indicated time points. $n = 9$ –14/group. (E) Representative H&E-stained colon sections are shown. Scale bars: 200 μ m. Histologic inflammatory scores were determined for each section from DSS-induced mice treated with/without YO-2. $n = 3$ /group. Values represent means \pm SEM. * $P < .05$, ** $P < .01$, and *** $P < .001$, determined by a 2-tailed Student t test and log-rank test.

of 2.5% TNBS (Sigma, St. Louis, MO) dissolved in 50% ethanol. The control group received only 50% ethanol. Anti-CD40 antibody (Ab) (clone FGK45; kindly provided by Dr Yagita, Department of Immunology, Juntendo University School of Medicine).^{15,16}

Trans-4-aminomethylcyclohexanecarbonyl-Tyr(*O*-Pic)-octylamide (YO-2)¹⁷ (kindly provided by Yoshio Okada, The Faculty of Pharmaceutical Science, Kobe Gakuin University) was administered by intraperitoneal injection, once per day at 4 mg/kg/day from days 0 to 28. The tranexamic acid moiety of YO-2 interacts with the active center of plasmin. The MMP inhibitor (N-hydroxy-2-[(4-methoxysulfonyl)(3-picolyl)-amino]-3-methylbutanamide [MMI270]) (Novartis Pharma Corporation, Basel, Switzerland)¹⁸ was administered orally at a dose of 100 mg/kg/day from days 0 to 7.

CD11b neutralizing antibody treatment. Mice were injected with 500 μ g/mouse of anti-CD11b antibody (Ab) (clone 5C6; kindly provided by Dr Yagita, Juntendo University) or control IgG on days 0, 2, and 4. The clinical scoring of the disease activity index (DAI) for DSS-induced colitis was based

on weight loss, stool consistency, and bleeding, as described previously by Cooper et al.¹⁹

Histology

Colon sections were stained with H&E and Elastica van Gieson staining. Histologic scoring was performed as described by Cooper et al.¹⁹

Bleeding Time

The mouse's tail was warmed to 37°C, and amputated 1 cm from the tail tip. Blood was blotted onto filter paper every 15 seconds.

Immunohistochemistry

Colon sections were stained with the anti-Gr1 Ab (clone RB6-8C5; R&D Systems, Minneapolis, MN), anti-MMP9 Ab (R&D Systems), anti-F4/80 Ab (clone A3-1; AbD Serotec, Oxford, United Kingdom), biotin-conjugated anti-CD11b Ab (clone M1-70; BD Pharmingen, San Diego, CA), anti-uPA Ab (Proteintech, Chicago,

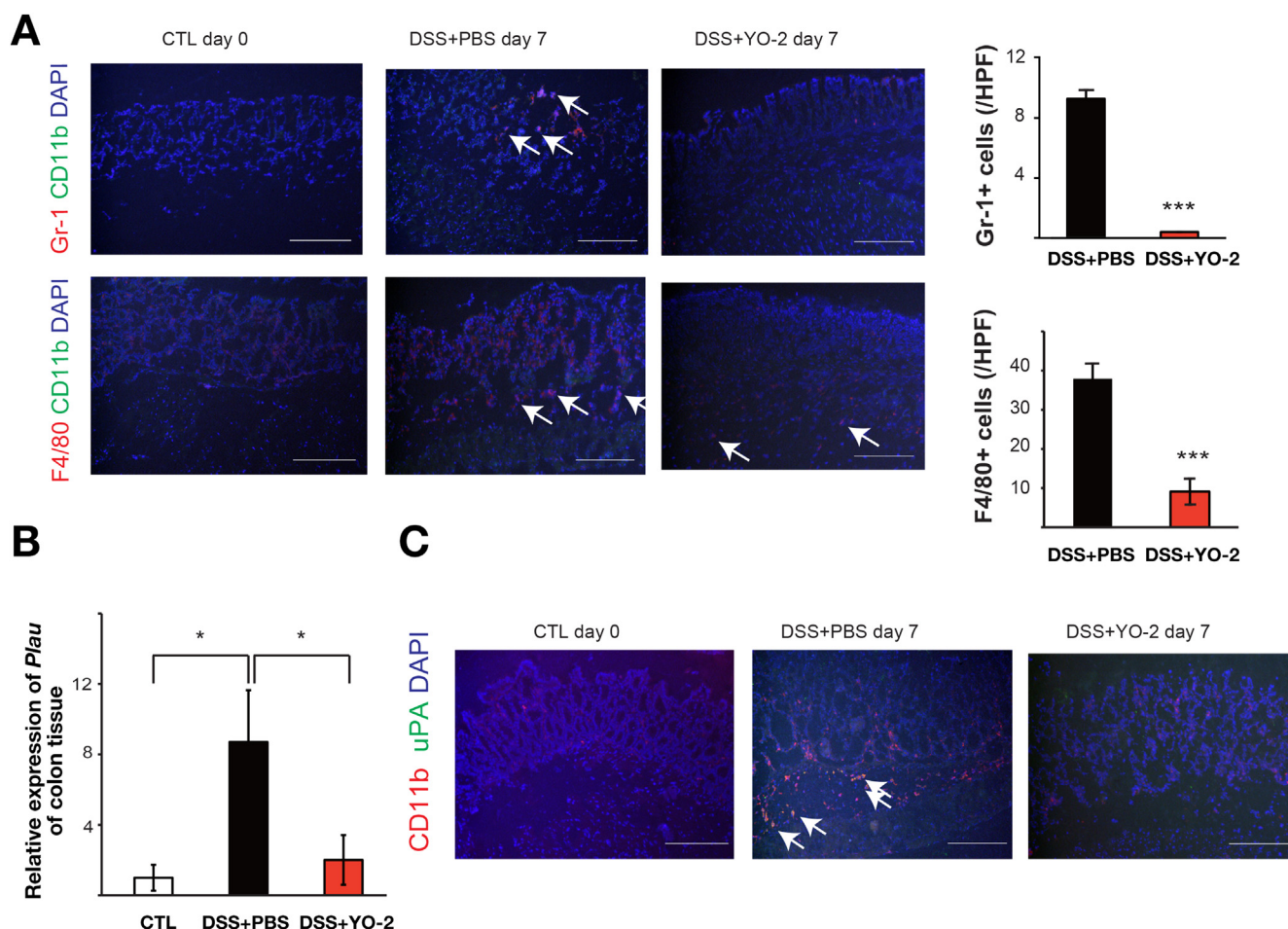


Figure 2. Plasmin inhibition suppresses the influx of myeloid cells into colonic tissue. (A) Representative immunohistochemical images of Gr-1/CD11b- and F4/80/CD11b-stained colon sections retrieved 7 days after DSS induction of C57BL/6 mice treated with or without YO-2. Arrows indicate positively stained cells. $n = 3$ /group. Scale bars: 200 μ m. Right: Indicated cell populations were quantified per high-power field (HPF). (B) *Plau* gene expression in colon retrieved from mice treated with or without YO-2 was semiquantitated by polymerase chain reaction. Levels were normalized to *Actb*. $n = 3$ /group. (C) Staining of colonic sections showed uPA staining of a CD11b⁺ cell subpopulation. Scale bars: 200 μ m. Data represent means \pm SEM. Arrows indicate double positive cells $n = 3$ group. * $P < .05$ and *** $P < .001$, determined by a 2-tailed Student *t* test. DAPI, 4',6-diamidino-2-phenylindole.

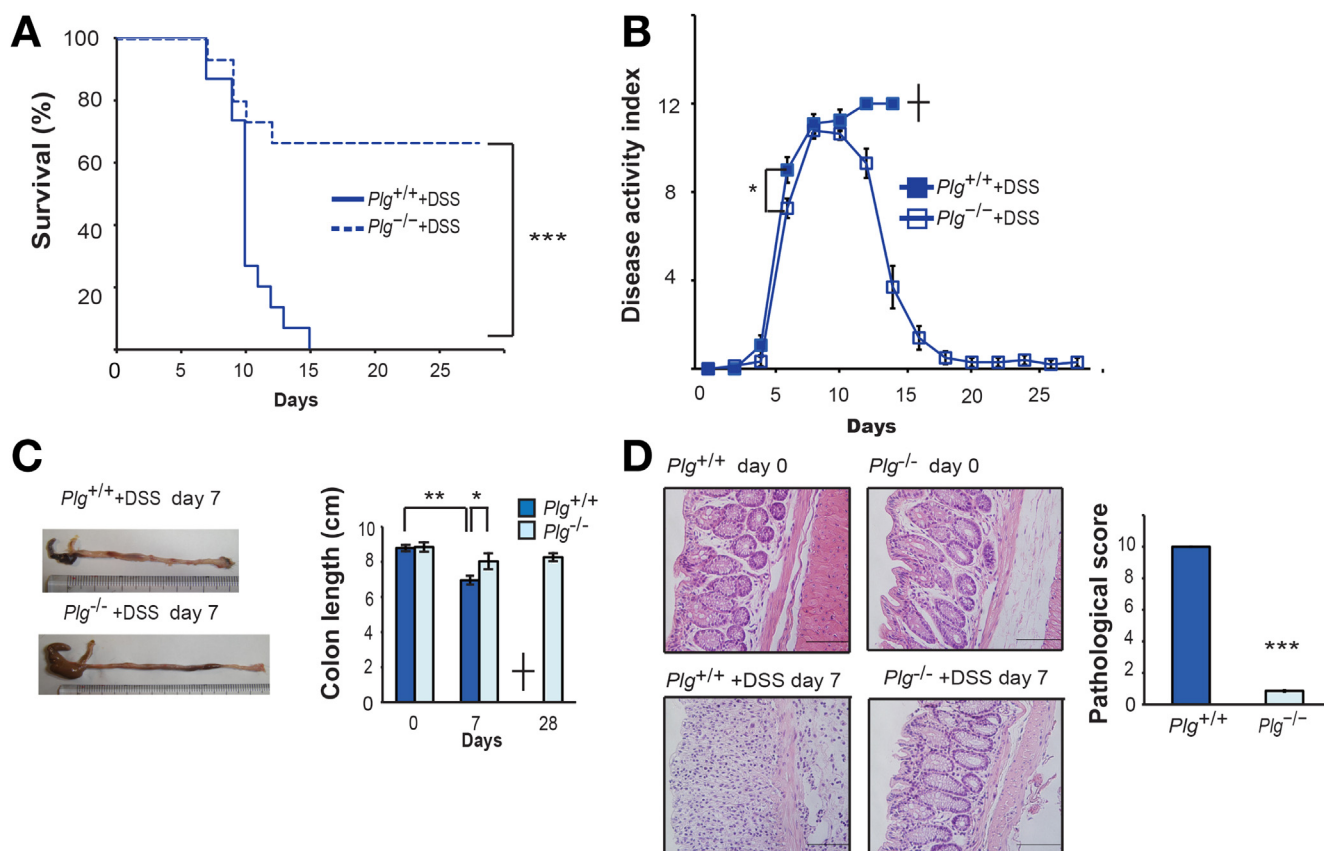


Figure 3. *Plg*-deficient mice are less susceptible to DSS-induced colitis. (A) Survival and (B) DAI were determined over the indicated time period in DSS-treated $Plg^{+/+}$ and $Plg^{-/-}$ mice: $n = 15$ /group. (C) $Plg^{-/-}$ mice show shorter colon lengths. $n = 9$ for each group. Right: Representative H&E-stained colon sections from DSS-treated $Plg^{+/+}$ and $Plg^{-/-}$ mice. Scale bars: 200 μ m. $n = 3$ /group for all experiments. (D) Right: Pathologic scores obtained using colon tissues. Values represent means \pm SEM. The percentage of survival was determined. $n = 3$. * $P < .05$, ** $P < .01$, and *** $P < .001$, determined by a 2-tailed Student t test and log-rank test.

IL), and antifibrinogen Ab (Biogenesis, Poole, United Kingdom) followed by donkey anti-rat IgG Ab conjugated with 594, donkey anti-rabbit, or goat IgG Ab conjugated with Alexa 488 and streptavidin-Alexa 488 (Invitrogen, Carlsbad, CA). Nuclei were stained using 4',6-diamidino-2-phenylindole.

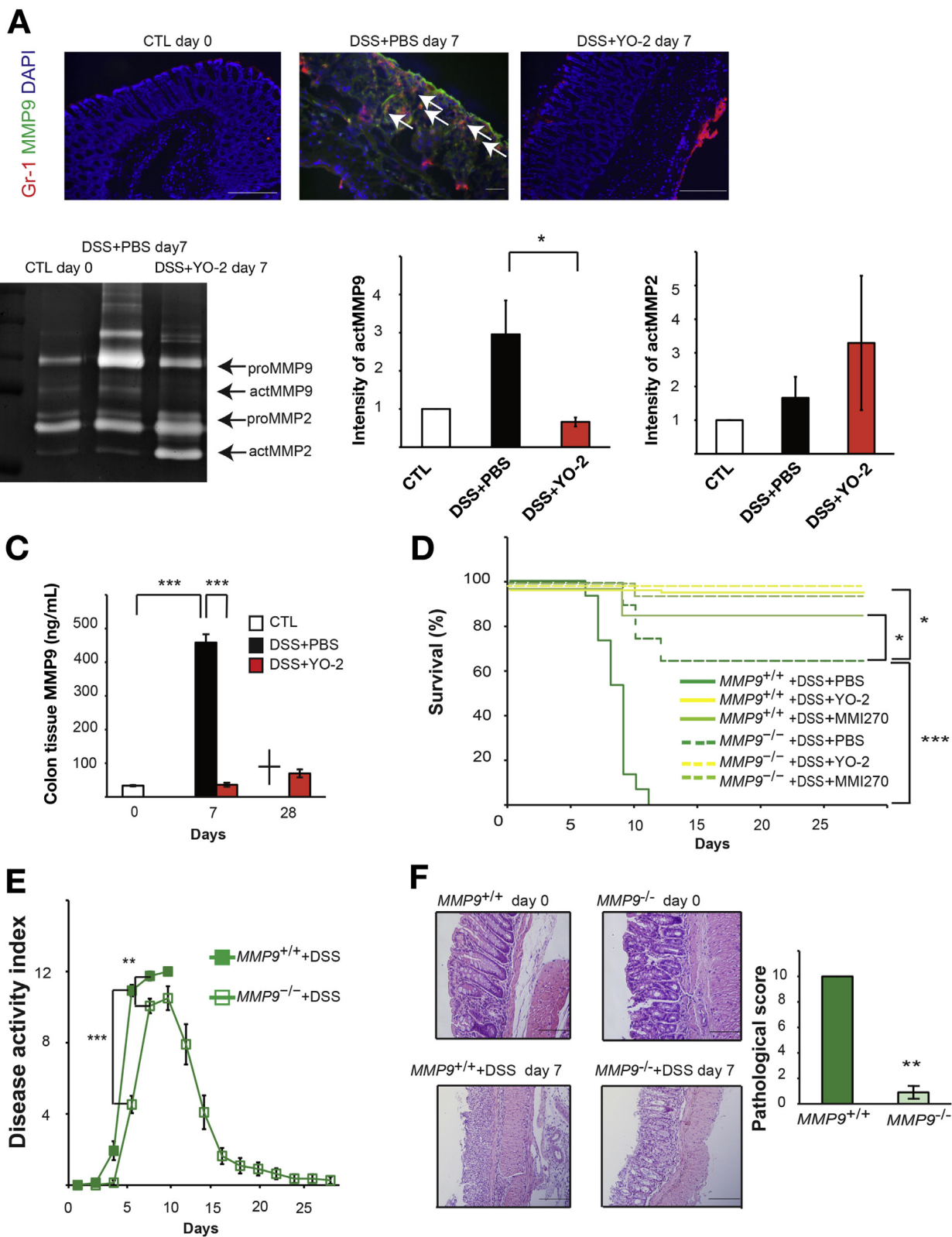
Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

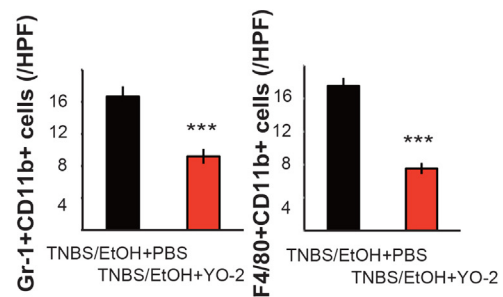
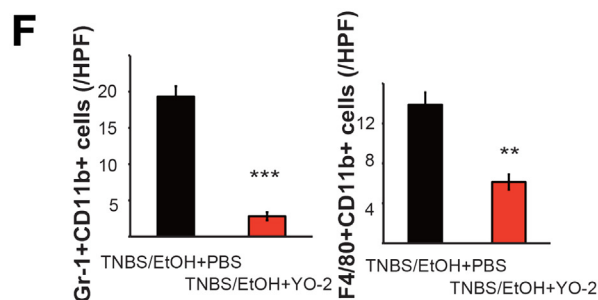
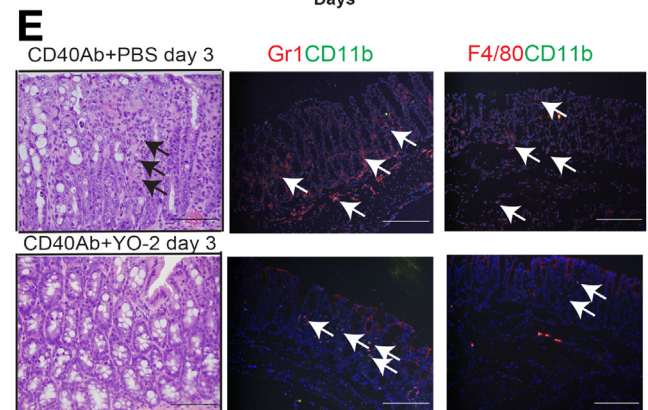
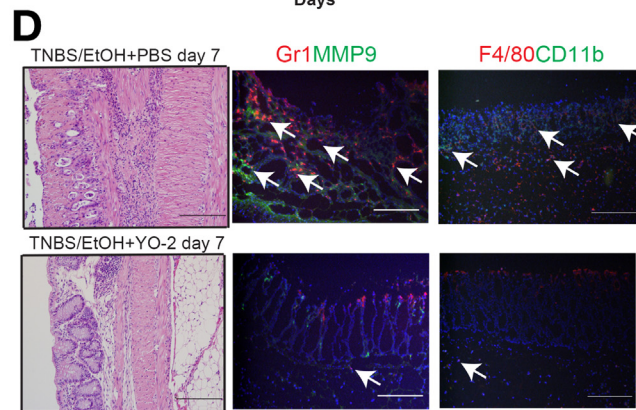
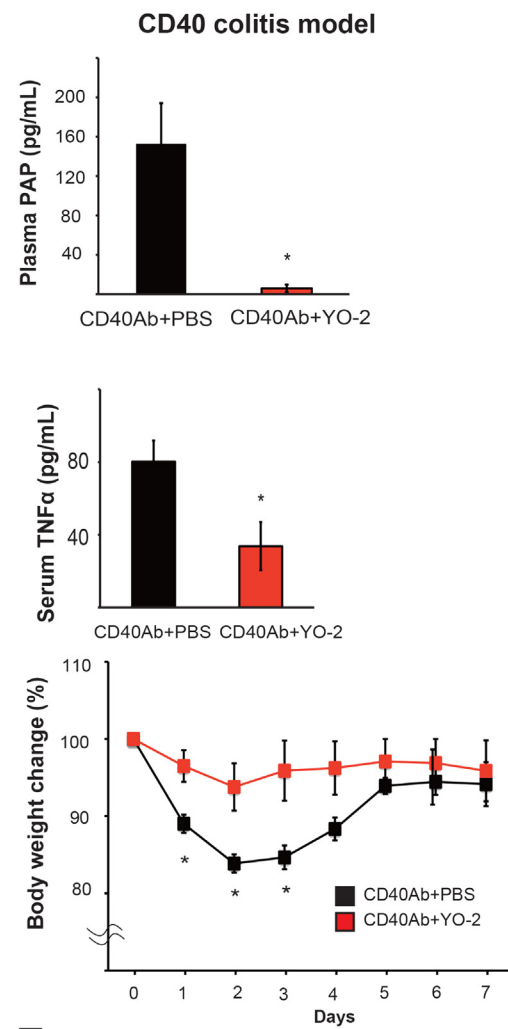
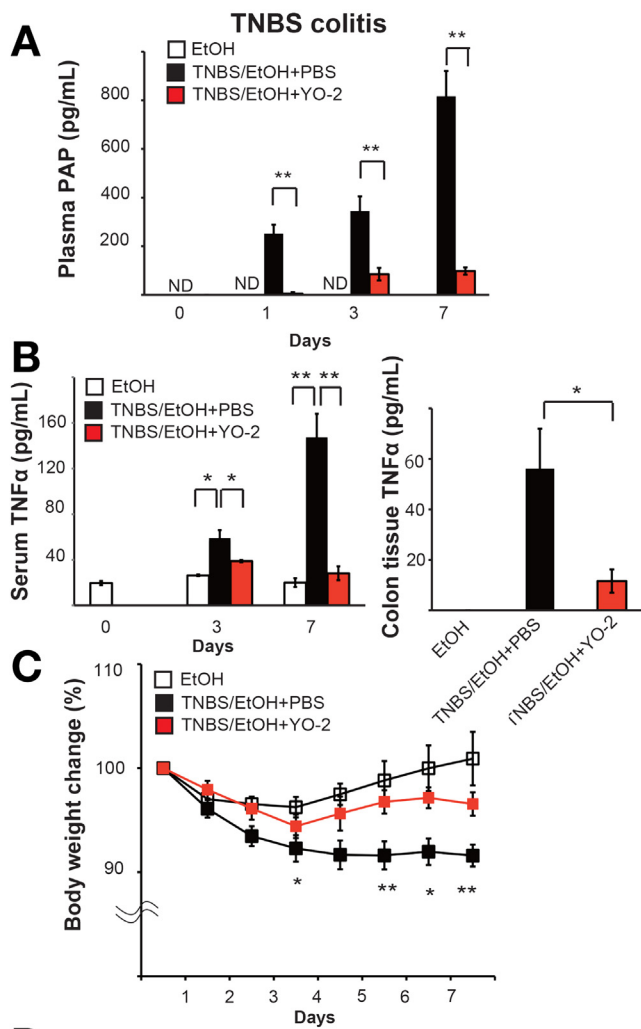
Apoptosis was detected using colon sections according to the instructions provided by the manufacturer of the TAC2 TdT Kit (Funakoshi, Tokyo, Japan).

Quantification of Cytokine Messenger RNA Expression in Colon Tissues by Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Invitrogen), and complementary DNA was generated according to the manufacturer's protocols. A total of 10 mg of colon was used. Peripheral blood mononuclear cells were isolated by centrifugation using Lympholyte (Cedarlane, Inc, Ontario, Canada). Polymerase chain reaction was performed with the following specific forward and reverse primer pairs, respectively: *Plau*: 5'-gtcctctctgcaacagagtc-3' and 5'-ctgtgtctgagggtaatgct-3'; *Plat*: 5'-gtactgcttggact-3' and 5'-tgctgttggaagtgtctg-3'; *Tnf*: 5'-gccgatttgctatctacac-3' and

Figure 4. Plasmin-mediated prevention of colitis progression requires MMP9. (A) Representative immunohistochemical images of Gr-1/MMP9-stained colon tissue sections retrieved 7 days after DSS induction of C57BL/6 mice treated with or without YO-2. Arrows indicate positively stained cells. $n = 3$ /group. (B) Blood serum samples and (C) culture supernatants derived from colon tissue of YO-2- or phosphate-buffered saline (PBS)-treated C57BL/6 mice were analyzed by gelatinolytic zymography (left). Right: Quantification of the proteolytic activity of MMP2 and MMP9 were detected by densitometry. $n = 5$ /group. (D) Survival was determined in DSS-induced colitic $Mmp9^{-/-}$ and wild-type mice injected with YO-2 or MMI270. Values represent means \pm SEM. Survival was determined in the following treatment groups: $Mmp9^{+/+}$ + PBS and $Mmp9^{+/+}$ + YO-2, $n = 15$; $Mmp9^{-/-}$ + PBS and $Mmp9^{-/-}$ + YO-2, $n = 15$; $Mmp9^{+/+}$ + MMP270 and $Mmp9^{-/-}$ + MMP270, $n = 20$. (E) DAI and representative H&E-stained colon sections (F) from DSS-treated $Mmp9^{+/+}$ and $Mmp9^{-/-}$ colitic mice (right panel) are shown. Scale bars: 200 μ m. $n = 3$ /group for all experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$, determined by a 2-tailed Student t test and log-rank test. CTL, control day 0.





5'-ggatatgggctcataccag-3'; *Il1b*: 5'-gcaactgttcctgaactc-3' and 5'-ctcggagcctgtagtgc-3'; *Il6*: 5'-tgacaggatgcagaaggaga-3' and 5'-gtcgaaggtggacagtgc-3'; *Cxcl5*: 5'-gcatttctgtgtgtcacgtg-3' and 5'-cctcctctggttttcagtttagc-3'; *Actb* control: 5'-gtatgaacacgatgatg-3' and 5'-ccagaagaccagagaaa-3'.

Immunoassay

The levels of cytokines were determined in plasma, serum, and colon supernatants. Colon supernatants were prepared as described previously.²⁰ Samples were measured using mouse-specific enzyme-linked immunosorbent assay kits for murine MMP9, CXCL5, TNF- α (all R&D Systems), plasmin-antiplasmin complex (PAP) (Cusabio Biotech, Newark, DE), fibrin degradation product (FDP) (Uscn Life Science, Inc, Wuhan, China), thrombin-antithrombin (Abcam, Cambridge, MA), and interleukin (IL)1 β and IL6 (Biolegend, San Diego, CA).

Gelatin Zymography

Plasma samples were treated with 20 μ L gelatin-agarose beads at 4°C overnight and processed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis acrylamide gels containing 1 mg/mL⁻¹ gelatin. For more detail see the article by Heissig et al.²¹

Transwell Migration Assay

Serum-starved U937 cells (1×10^6) were placed in 24-well Transwell inserts with 8- μ m pores (Corning Life Sciences, Lowell, MA). Cells were allowed to migrate for 2 hours at 37°C toward colon supernatants derived from DSS-induced mice treated with or without YO-2 in the presence of IgG and CXCL5 Ab, or RPMI-1640 medium alone. The number of migrated cells was counted.

Statistical Analyses

All data are presented as means \pm SEM. Student *t* tests were performed. Survival curves were plotted using Kaplan-Meier estimates with log rank. A *P* value less than .05 was considered significant.

Results

Plasmin Is Activated During the Early Phase of Experimental Colitis

We investigated whether fibrinolytic factors are present during the progression of IBD by using DSS-induced colitis as a model. An increase in PAP, a measure of active plasmin,

was detected in plasma of treated mice, peaking 3 days after the onset of DSS treatment as determined by enzyme-linked immunosorbent assay (Figure 1A). We next evaluated the effects of the plasmin inhibitor YO-2 administration on fibrinolytic and coagulation factors. YO-2 blocks the catalytic site of plasmin, and thereby efficiently inhibits active circulating plasmin.²² YO-2 treatment inhibited the systemic increase in PAP in plasma within the colon of DSS-treated animals (Figure 1A). Clinically, IBD patients often show both excessive fibrinolysis and coagulation.²³ Because the function of plasmin is to dissolve fibrin clots, plasmin inhibition might alter coagulation and fibrin deposition/clot formation. However, YO-2 prevented the increase in the coagulation marker thrombin-antithrombin on day 7 (Supplementary Figure 1). Bleeding time was impaired in both phosphate-buffered saline- and YO-2-treated colitic mice (Supplementary Figure 2). No fibrin(ogen) staining pattern was observed in colitic tissues with or without YO-2 on day 7 and YO-2-treated normal colon tissues for 28 days (Supplementary Figure 3). When plasmin breaks down fibrin, FDPs are produced. No significant change in plasma FDP levels was observed in blood samples of DSS-induced colitic mice treated with or without YO-2 (Supplementary Figure 4). The data indicated that a plasmin increase occurs during the early phase of colitis, but no signs of fibrin deposition were found.

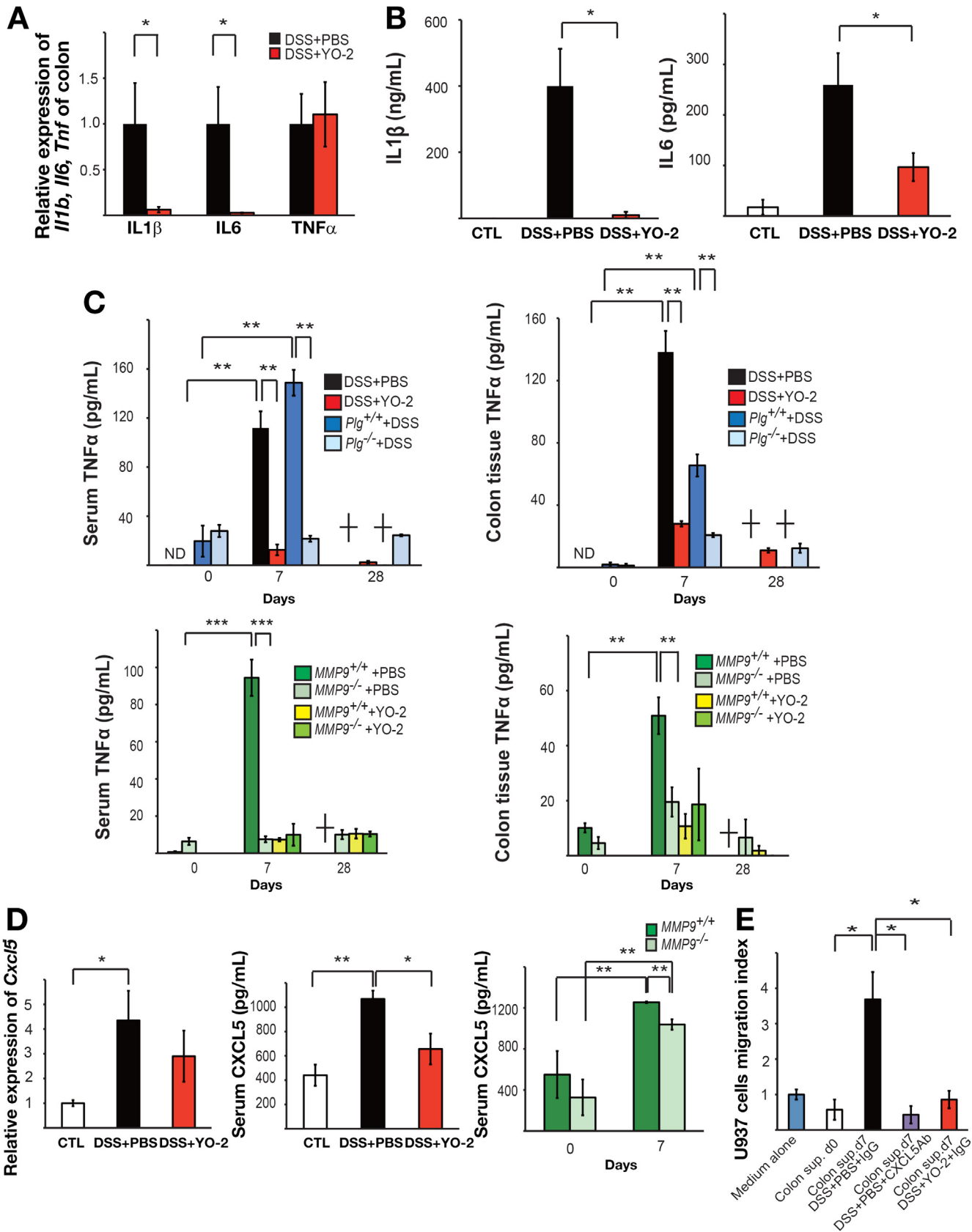
Pharmacologic Targeting of Plasmin Prevents DSS-Induced Colitis Progression

Plasmin inhibition by YO-2 treatment protected against DSS-induced colitis-associated lethality, and ameliorated the high DAI (a numeric value reflecting weight loss, diarrhea, and bleeding), and prevented shortening of the colon (Figure 1B-D). In line with these observations, colon sections of YO-2-treated mice showed less severe mucosal damage, loss of goblet cells, and inflammatory cell infiltration (Figure 1E), indicating that plasmin activation contributes to colitis progression.

Plasmin Regulates uPA-expressing Myeloid Cell Influx Into Colonic Tissues

Plasmin inhibition prevented the recruitment of Gr-1⁺ CD11b⁺ neutrophils and F4/80⁺ CD11b⁺ macrophages into the mucosal region of the colon (Figure 2A). *Plau*

Figure 5. Plasmin inhibition ameliorates TNBS- and CD40-induced colitis. Colitic mice induced by TNBS administration (*left*) or by CD40 Ab injection into *Rag2*^{-/-} mice (*right*) were treated with phosphate-buffered saline (PBS) and YO-2. Levels of PAP in (A) plasma and (B) TNF- α in serum and in colon supernatants of indicated experimental groups by enzyme-linked immunosorbent assay. (C) Body weight loss, with starting body weight set as 100% in control and TNBS- and CD40-treated mice. *n* = 10/group. (D) Representative images of colon sections from TNBS-induced colitic mice stained with H&E, and immunostained using Gr-1, MMP9, and F4/80 Abs. Scale bars: 200 μ m. (E) Representative images of colon sections from CD40 Ab-induced colitic mice stained with H&E, and immunostained using Gr-1, CD11b, and F4/80 Abs. Scale bars: 100 μ m. YO-2 prevents the infiltration of Gr-1⁺ neutrophils and F4/80⁺ macrophages (white arrows). (F) Quantification of indicated cell populations in indicated treatment groups, *n* = 3. Data represent means \pm SEM. **P* < .05, ***P* < .01, and ****P* < .001, determined by a 2-tailed Student *t* test. HPF, high-power field.



(Figure 2B), but not *Plat* (data not shown), expression was up-regulated in colon tissues of DSS-treated mice. In accordance with previous studies,²⁴ YO-2 treatment prevented the influx of uPA-co-expressing CD11b⁺ cells in colonic tissues (Figure 2C). These data suggest that local myelomonocytic cells are a source of uPA during colon inflammation.

Reduced Disease Severity in *Plg*^{-/-} Mice With DSS-Induced Colitis

To further study the role of *Plg* in DSS-induced colitis progression, *Plg*^{-/-} mice were used. *Plg*^{-/-} mice were protected from DSS-induced colitis, showing a lower mortality rate (Figure 3A), a lower DAI (Figure 3B), and diminished colon length shortening (Figure 3B and C). Because colon shortening might be owing to increased cell apoptosis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining on colon sections was used to determine apoptosis. Fewer terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling-positive cells were observed in *Plg*^{-/-} mice, indicating that *Plg* deficiency protects from apoptosis in colitic mice (Supplementary Figure 5). *Plg*^{-/-} mice showed reduced histologic signs of inflammation (Figure 3D). Less deposition of collagen and elastic fiber as stained by Elastica van Gieson was observed in *Plg*^{-/-} colon sections compared with *Plg*^{+/+} mice (Supplementary Figure 6). These results suggest that plasmin accelerates intestinal inflammation, which might result in increased intestinal apoptosis leading to fibrosis, and, ultimately, causing colon shortening.

Plasmin Enhances Colitis Progression in a Partly MMP9-Dependent Manner

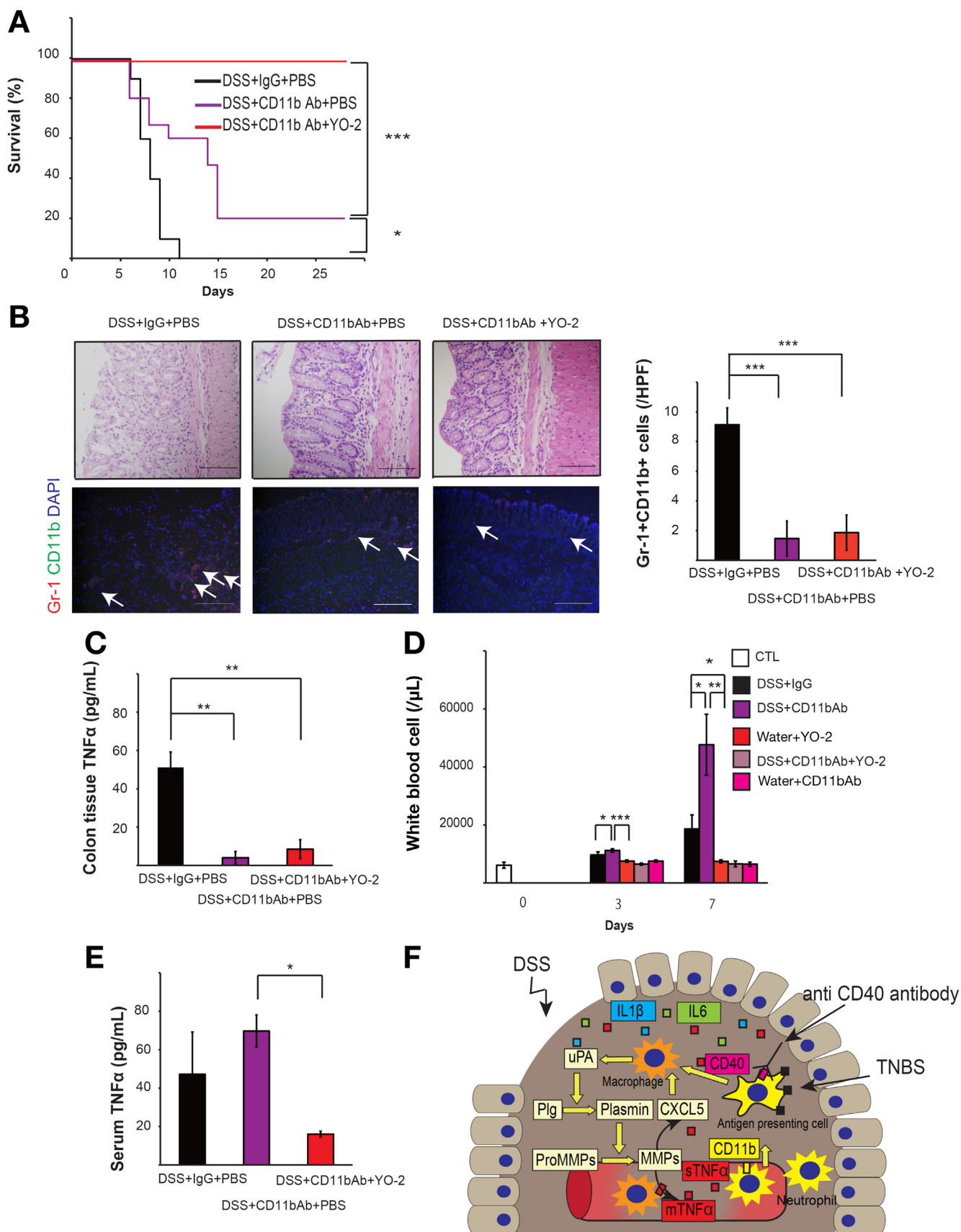
Because plasmin can activate MMPs, and MMPs such as MMP3, MMP7, and MMP12 are linked to the progression of colitis,²⁵ we hypothesized that plasmin, by activating proteases such as MMP9, might contribute to colitis progression in vivo. Immunoreactive MMP9 was observed in epithelial cells and neutrophils of colon sections derived from DSS-induced colitic mice, but not in YO-2-treated

mice (Figure 4A). Similarly, YO-2 treatment prevented the increase in total serum MMP9 in YO-2-treated DSS-induced colitic mice (Supplementary Figure 7), with a decrease in both the pro-MMP and active form of MMP9, but not MMP2, as determined by zymography (Figure 4B). Similarly, colon supernatants of YO-2-treated DSS-induced colitic mice contained less total MMP9 (Figure 4C). DSS-induced colitic *Mmp9*^{-/-} mice showed an improved survival rate, a lower DAI, prevented infiltration of inflammatory cells, and colonic tissue destruction (Figure 4D–F). In comparison with deletion of MMP9 alone, the broad-spectrum MMP inhibitor MMI270, which inhibits several MMPs, ameliorated colitis progression to a greater extent (Figure 4D). However, when comparing YO-2- and MMI270-treated mice, YO-2-treated mice showed the best survival rate because 10% of MMI270-treated mice died after suffering from a musculoskeletal syndrome, a known side effect of MMP inhibitors containing a hydroxamate structure.²⁶ No side effects were observed in YO-2-treated mice.

Plasmin Inhibition Ameliorates Colitis After TNBS and CD40 Induction

We extended our analysis by evaluating the effect of plasmin inhibition in 2 other colitis models: the TNBS-induced colitis model and the anti-CD40 colitis model. In YO-2-treated colitic mice, no increase in circulating plasmin level and no augmentation in colon-derived or circulating TNF- α level was observed (Figure 5A and B). YO-2 treatment prevented colitis-related body weight loss (Figure 5C) and the destruction of the colonic epithelium in both colitis models (Figure 5D and E). Immunoreactive MMP9 was found in the colonic tissue of phosphate-buffered saline-treated, but not YO-2-treated, mice in TNBS colitis (Figure 5D). In addition, YO-2 treatment reduced the number of infiltrating inflammatory Gr-1⁺ and F4/80⁺ myeloid cells (Figure 5F). These data suggest that plasmin inhibition suppressed clinical progression of the colitis-associated cytokine storm in 3 murine models of colitis.

Figure 6. Pharmacologic plasmin inhibition alleviates systemic and colonic cytokine production in DSS-induced colitic mice. (A) Gene expression of *Il1b*, *Il6*, and *Tnf* in colon homogenates of C57BL/6 mice treated with phosphate-buffered saline (PBS) or YO-2 as determined by polymerase chain reaction (normalized to the expression of *Actb*). n = 4 or 5/group. (B) The inflammatory cytokines IL1 β and IL6 were assayed in serum samples of mice with DSS-induced colitis treated with or without YO-2 as determined by enzyme-linked immunosorbent assay. n = 6/group. (C) TNF- α levels were measured in serum samples (left panel) or in supernatants (right panel) of cultured colon tissue retrieved from YO-2- or PBS-treated C57BL/6 mice, *Plg*^{+/+} or *Plg*^{-/-}, and *Mmp9*^{+/+} and *Mmp9*^{-/-} mice 7 days after the initiation of DSS treatment by enzyme-linked immunosorbent assay. n = 3–6/group. (D) CXCL5 expression in colon samples extracted from DSS-induced colitic mice treated with or without YO-2 (left panel). CXCL5 levels in serum of YO-2- or PBS-treated C57BL/6 mice (middle panel) and *Mmp9*^{-/-} (right panel), or their respective littermate controls as determined by enzyme-linked immunosorbent assay. n = 3–6/group. (E) Transmigration index of U937 cells migrating toward medium alone, colon supernatants extracted from normal mice (d0), or supernatants from DSS-treated mice injected with PBS or YO-2 in the presence or absence of CXCR5 Ab. n = 3–5/group. Data represent means \pm SEM. *P < .05 and **P < .01, determined by a 2-tailed Student t test. CTL, control day 0.



Plasmin and Mmp9 Deficiency Reduce the Cytokine Storm Associated With Colitis

We next examined typical IBD-associated cytokines in DSS-induced colitis. The expression of *Il1b* and *Il6* messenger RNA (Figure 6A) in colonic extracts and circulating IL1 β and IL6 (Figure 6B) was lower in YO-2-treated colitic mice. Plasmin deletion or inhibition and *Mmp9* deficiency prevented the increase in circulating TNF- α serum levels and suppressed TNF- α release from colonic supernatants after DSS induction (Figure 6A–C). Although MMP9 is a downstream target of plasmin, plasma PAP levels were low in colitic *Mmp9*^{-/-} mice (Supplementary Figure 8) by preserving (eg, crypt destruction and epithelial cell sloughing). These data indicate that plasmin is important in mounting a cytokine response.

Reduced CXCL5 Release During Colitis After Plasmin and MMP9 Inhibition

In vivo processing of CXCL5 by MMP2 and MMP9 promotes neutrophil recruitment in a model of peritonitis.²⁷ Although YO-2 treatment did not alter *Cxcl5* gene expression significantly, in YO-2-treated mice and *MMP9*^{-/-} mice no increase in serum CXCL5 levels was observed (Figure 6D). We investigated whether CXCL5 is released from colonic tissues and might be involved in leukocyte migration. The improved myeloid (U937) cell migration toward supernatants derived from colitic mice was the result of plasmin/MMP9-dependent production of CXCL5, as shown using neutralizing Abs (Figure 6E). These data show that plasmin enhances the colon-derived release of CXCL5, thereby enhancing neutrophil influx.

CD11b⁺ Cells Drive Plasmin-Mediated Colitis Progression

If the recruitment of CD11b⁺ cells is the main function of plasmin-mediated effects during colitis, blocking CD11b cell recruitment using CD11b neutralizing Abs should improve clinical signs of colitis. However, this was not the case (Figure 7A). Similar to previous reports,²⁸ 80% of

DSS-induced mice treated with neutralizing Abs against CD11b died (Figure 7A), although CD11b⁺ cell influx into colonic tissues (Figure 7B) and local colonic TNF- α production was blocked (Figure 7C). Mononuclear cells isolated from the blood of CD11b Ab-treated, but not YO-2-treated, mice showed high *Tnf* messenger RNA expression (Supplementary Figure 9). A systemic TNF- α increase correlated with leukocytosis by mononuclear cells, whereas local colonic TNF- α production correlated with the number of infiltrated CD11b⁺ cells (Figure 7C–E). Survival, white blood cell counts, histopathologic changes, and TNF- α serum level increase were suppressed after co-injection of YO-2 with CD11b Abs (Figure 7A–E). Our data suggest that plasmin promotes colitis progression by accelerating leukocytosis and leukocyte activation, resulting in the release of TNF- α , and by enhancing the infiltration of myeloid cells into colonic tissues.

Taken together, our data indicate that the activation of the fibrinolytic pathway accelerates the inflammatory response during colitis progression by the influx of CD11b⁺ myeloid cells.

Discussion

In this study, we provide genetic, functional, and biochemical evidence that the orderly activation of 2 separate protease systems, the fibrinolytic system and the MMP system during experimental colitis, drives mucosal inflammation, a process in part mediated by an accelerating systemic and colonic increase in CD11b⁺ inflammatory cells and the release of the proinflammatory cytokine TNF- α and of the chemokine CXCL5. Collectively, these data introduce a novel paradigm by which fibrinolytic enzymes mediate systemic and localized effects in the colonic tissues and establish a novel role for Plg activation in colitis. We recently showed that addition of TNF- α to myeloid cells increased the expression of uPA in vitro.²⁹ We found local up-regulation of the uPA in inflamed, but not control, colonic tissues. The observed low uPA expression in colitic tissues in plasmin inhibitor-treated mice could be owing to the impaired infiltration of uPA-producing CD11b⁺ cells and/or

Figure 7. Improved disease control and inflammatory cytokine response in YO-2-treated compared with CD11b Ab-treated mice during experimental colitis. DSS-induced C57BL/6 mice were treated with YO-2 and co-injected with anti-CD11b or control Abs. (A) The survival rate was determined in the following treatment groups: 2% DSS + IgG + phosphate-buffered saline (PBS), n = 10; 2% DSS + CD11b Ab + PBS, n = 15; 2% DSS + CD11b Ab + YO-2, n = 10. (B) Representative H&E-stained colon sections from treated mice. Scale bars: 200 μ m. (C) TNF- α protein in supernatants from colon cultures at day 7. (D) White blood cells were counted at the indicated time points. n = 6/group. (E) TNF- α protein was determined in plasma samples taken at day 7. Dates represent means \pm SEM. **P* < .05, ***P* < .01, and ****P* < .001, determined by a 2-tailed Student *t* test and log-rank test. (F) A model of various target molecules of plasmin involved in experimentally induced colitis. The secreted plg is processed into its enzymatically active form plasmin by uPA, which is supplied by the activated CD11b⁺ inflammatory cells during colitis. Plasmin in turn sensitizes macrophages and/or epithelium to accelerate the conversion from pro-MMP9 to MMP9. The proteolytic environment generated not only damage to colonic tissues, but also releases proinflammatory cytokines (eg, TNF- α) and chemokines (eg, CXCL5) known to promote the influx of CD11b⁺ cells into colonic tissues. These CD11b⁺ cells promote colonic tissue damage in part by again providing necessary proteases to fuel this vicious cycle. DAPI, 4',6-diamidino-2-phenylindole.

the blockade of local and systemic TNF- α increase. Our data showing the PAP increase in DSS-treated mice are in accordance with clinical reports from patients with IBD.³⁰ The presence of fibrinolytic factors within colonic tissues (eg, uPA-expressing myeloid cells),²⁴ suggest that localized proteolysis occurs during colitis, paving the way for inflammatory cells or intestinal microbes to further induce tissue damage. Our data are shown in Figure 7F.

The fibrinolytic system is activated in IBD patients; this is especially true for patients with active disease.^{31,32} Our initial decision to explore the therapeutic potential of YO-2 was motivated by previous studies showing that administration of the antifibrinolytic agents ϵ -aminocaproic acid and tranexamic acid improved clinical outcomes of patients with UC.^{33,34} Here, we show that plasmin inhibition enabled recovery from colitis-induced tissue damage and improved clinical outcomes.

What are the potential downstream targets of plasmin during the colitis process? We were able to show that the activation of MMPs such as MMP9 is a critical downstream event during colitis progression. Plasmin has been shown to activate MMP9 in other disease models, including nerve injury,²⁸ disease progression in acute graft-versus-host disease after bone marrow transplantation,²⁹ and hematopoietic and ischemic tissue regeneration.^{7,9} It has been well established that MMP9 is up-regulated consistently in both animal models and human IBD, and is associated with disease severity.^{3,35} Confirming data by Castaneda et al,³ we showed that *MMP9*^{-/-} mice exposed to DSS showed reduced colitis severity. Epithelial MMP9 but not infiltrated neutrophil-derived MMP9, has been shown to induce tissue damage. Here, we found that plasmin inhibition prevented MMP9 activation and TNF- α release both systemically and locally in the colonic environment. TNF- α release involves a process called shedding, which involves the protease ADAM17/TNF- α converting enzyme, but also other MMPs such as MMP1, MMP7, and MMP9.³⁶ Our data indicate that plasmin-dependent TNF- α production requires endogenous MMP9, which promotes TNF- α production either directly or indirectly by activating other proteases. However, YO-2 also seems to have MMP9-independent modes of action because YO-2 treatment improved survival even in *Mmp9*^{-/-} mice. Because plasmin can activate other MMPs such as MMP3, MMP7, and MMP12, which are linked to the progression of colitis,²⁵ we suspect that the control of other MMPs might be responsible for the improved survival of *Mmp9*^{-/-} mice treated with YO-2.

Anti-inflammatory medication is a mainstay of IBD treatment. Infliximab, a monoclonal Ab to TNF- α , appeared to be a good therapeutic agent for IBD patients.³⁷ However, aside from its high expense, Ab treatment can lead to infusion reactions, loss of response, and serum sickness.³⁸ Use of small molecules such as the plasmin inhibitor not only would be cheaper, but might even be safer because there is

less risk of immunogenicity in patients. Furthermore, YO-2 could prevent the other inflammatory cytokines IL1 β and IL6.

The influx of CD11b⁺ macrophages and neutrophils into the inflamed tissue is a critical pathogenic aspect of colitis in a plasmin-dependent manner. CD11b interacts with the intercellular adhesion molecules intercellular adhesion molecules 1 and 2,³⁹ which have been suggested as therapeutic targets in colitis. Although CD11b Ab treatment slightly improved survival in colitic mice, the Abs could not control systemic TNF- α release, so that peripheral leukocytosis proceeded unabated. These data are consistent with that of other inflammatory models, such as peritonitis, in which CD11b Ab treatment prevented myelomonocytic cell recruitment in vivo.⁴⁰

We previously showed that plasmin regulates Gr-1⁺ neutrophil infiltration during recovery from hindlimb ischemia.⁸ Here, we found that plasmin inhibition prevented the infiltration of Gr-1⁺ neutrophils into colonic tissues. Enterocyte-derived CXCL5 can attract CXCR2⁺ neutrophils into the gut tissues.⁴¹ Recently, it was shown that in vivo processing of CXCL5, MMP2, and MMP9 promotes neutrophil recruitment in IL1 β -induced peritonitis.²⁷ Circulating CXCL5 was suppressed both in YO-2-treated mice and in *MMP9*^{-/-} mice, showing that YO-2 (most likely via MMP inhibition, however, further studies are necessary) can control CXCL5 production, thereby preventing influx of MMP9-carrying neutrophils.

Our data support a mechanism whereby activation of Plg during colitis progression leads to the activation of another protease cascade, namely MMPs. This proteolytic environment controls both cell infiltration into colonic tissues, as well as production and secretion of proinflammatory cytokines and chemokines. In contrast to TNF- α Abs, the targeting of plasmin can suppress the MMP cascade, thereby controlling the release of important proinflammatory cytokines. Our experimental approach here was prophylactic, but our findings suggest that YO-2 is an attractive candidate for targeting milder forms of IBD after onset.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2014.12.001>.

References

1. Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 2002;347:417–429.
2. Heissig B, Nishida C, Tashiro Y, et al. Role of neutrophil-derived matrix metalloproteinase-9 in tissue regeneration. *Histol Histopathol* 2010;25:765–770.
3. Castaneda FE, Walia B, Vijay-Kumar M, et al. Targeted deletion of metalloproteinase 9 attenuates experimental

- colitis in mice: central role of epithelial-derived MMP. *Gastroenterology* 2005;129:1991–2008.
4. Mao JW, He XM, Tang HY, et al. Protective role of metalloproteinase inhibitor (AE-941) on ulcerative colitis in rats. *World J Gastroenterol* 2012;18:7063–7069.
 5. Lijnen HR, Silence J, Lemmens G, et al. Regulation of gelatinase activity in mice with targeted inactivation of components of the plasminogen/plasmin system. *Thromb Haemost* 1998;79:1171–1176.
 6. Heissig B, Ohki-Koizumi M, Tashiro Y, et al. New functions of the fibrinolytic system in bone marrow cell-derived angiogenesis. *Int J Hematol* 2012;95:131–137.
 7. Ohki M, Ohki Y, Ishihara M, et al. Tissue type plasminogen activator regulates myeloid-cell dependent neoangiogenesis during tissue regeneration. *Blood* 2010;115:4302–4312.
 8. Tashiro Y, Nishida C, Sato-Kusubata K, et al. Inhibition of PAI-1 induces neutrophil-driven neoangiogenesis and promotes tissue regeneration via production of angiocrine factors in mice. *Blood* 2012;119:6382–6393.
 9. Heissig B, Lund LR, Akiyama H, et al. The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell Stem Cell* 2007;1:658–670.
 10. Black RA, Rauch CT, Kozlosky CJ, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature* 1997;385:729–733.
 11. Hattori K, Hirano T, Ushiyama C, et al. A metalloproteinase inhibitor prevents lethal acute graft-versus-host disease in mice. *Blood* 1997;90:542–548.
 12. Neurath MF, Fuss I, Pasparakis M, et al. Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. *Eur J Immunol* 1997;27:1743–1750.
 13. Doe WF, Dorsman B. Chronic inflammatory bowel disease—increased plasminogen activator secretion by mononuclear phagocytes. *Clin Exp Immunol* 1982;48:256–260.
 14. Souto JC, Martinez E, Roca M, et al. Low levels of plasminogen activator inhibitor type 1 in patients with inflammatory bowel disease. *Fibrinolysis* 1994;8:359–363.
 15. Uhlig HH, McKenzie BS, Hue S, et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 2006;25:309–318.
 16. Cayatte C, Joyce-Shaikh B, Vega F, et al. Biomarkers of therapeutic response in the IL-23 pathway in inflammatory bowel disease. *Clin Transl Gastroenterol* 2012;3:e10.
 17. Okada Y, Tsuda Y, Tada M, et al. Development of potent and selective plasmin and plasma kallikrein inhibitors and studies on the structure-activity relationship. *Chem Pharm Bull (Tokyo)* 2000;48:1964–1972.
 18. Peterson JT. Matrix metalloproteinase inhibitor development and the remodeling of drug discovery. *Heart Fail Rev* 2004;9:63–79.
 19. Cooper HS, Murthy SN, Shah RS, et al. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993;69:238–249.
 20. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. *Nat Protocols* 2007;2:541–546.
 21. Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 2002;109:625–637.
 22. Lee E, Enomoto R, Takemura K, et al. A selective plasmin inhibitor, trans-aminomethylcyclohexanecarbonyl-L-(O-picolyl)tyrosine-octylamide (YO-2), induces thymocyte apoptosis. *Biochem Pharmacol* 2002;63:1315–1323.
 23. van Bodegraven AA, Schoorl M, Linskens RK, et al. Persistent activation of coagulation and fibrinolysis after treatment of active ulcerative colitis. *Eur J Gastroenterol Hepatol* 2002;14:413–418.
 24. Vassalli JD, Dayer JM, Wohlwend A, et al. Concomitant secretion of prourokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. *J Exp Med* 1984;159:1653–1668.
 25. Naito Y, Takagi T, Kuroda M, et al. An orally active matrix metalloproteinase inhibitor, ONO-4817, reduces dextran sulfate sodium-induced colitis in mice. *Inflamm Res* 2004;53:462–468.
 26. Fisher JF, Mobashery S. Recent advances in MMP inhibitor design. *Cancer Metastasis Rev* 2006;25:115–136.
 27. Song J, Wu C, Zhang X, et al. In vivo processing of CXCL5 (LIX) by matrix metalloproteinase (MMP)-2 and MMP-9 promotes early neutrophil recruitment in IL-1 β -induced peritonitis. *J Immunol* 2013;190:401–410.
 28. Zou T, Ling C, Xiao Y, et al. Exogenous tissue plasminogen activator enhances peripheral nerve regeneration and functional recovery after injury in mice. *J Neuropathol Exp Neurol* 2006;65:78–86.
 29. Vrij AA, Rijken J, van Wersch JW, et al. Coagulation and fibrinolysis in inflammatory bowel disease and in giant cell arteritis. *Pathophysiol Haemost Thromb* 2003;33:75–83.
 30. Koutroubakis IE. Therapy insight: vascular complications in patients with inflammatory bowel disease. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:266–272.
 31. Kume K, Yamasaki M, Tashiro M, et al. Activations of coagulation and fibrinolysis secondary to bowel inflammation in patients with ulcerative colitis. *Intern Med* 2007;46:1323–1329.
 32. Salter RH, Read AE. Epsilon-aminocaproic acid therapy in ulcerative colitis. *Gut* 1970;11:585–587.
 33. Hollanders D, Thomson JM, Schofield PF. Tranexamic acid therapy in ulcerative colitis. *Postgrad Med J* 1982;58:87–91.
 34. Sato A, Nishida C, Sato-Kusubata K, et al. Inhibition of plasmin attenuates murine acute graft-versus-host disease mortality by suppressing the matrix metalloproteinase-9-dependent inflammatory cytokine storm and effector cell trafficking. *Leukemia* 2015;29:145–156.
 35. Kolho KL, Sipponen T, Valtonen E, et al. Fecal calprotectin, MMP-9, and human β -defensin-2 levels in pediatric inflammatory bowel disease. *Int J Colorectal Dis* 2014;29:43–50.
 36. Mohan MJ, Seaton T, Mitchell J, et al. The tumor necrosis factor- α converting enzyme (TACE): a unique

metalloproteinase with highly defined substrate selectivity. *Biochemistry* 2002;41:9462–9469.

37. Jarnerot G, Hertervig E, Friis-Liby I, et al. Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 2005;128:1805–1811.
38. Rutgeerts P, Van Assche G, Vermeire S. Optimizing anti-TNF treatment in inflammatory bowel disease. *Gastroenterology* 2004;126:1593–1610.
39. Sans M, Panes J, Ardite E, et al. VCAM-1 and ICAM-1 mediate leukocyte-endothelial cell adhesion in rat experimental colitis. *Gastroenterology* 1999;116:874–883.
40. Rosen H, Gordon S. Monoclonal antibody to the murine type 3 complement receptor inhibits adhesion of myelomonocytic cells in vitro and inflammatory cell recruitment in vivo. *J Exp Med* 1987;166:1685–1701.
41. Mei J, Liu Y, Dai N, et al. Cxcr2 and Cxcl5 regulate the IL-17/G-CSF axis and neutrophil homeostasis in mice. *J Clin Invest* 2012;122:974–986.

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Received January 14, 2014. Accepted December 2, 2014.

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Acknowledgments

The authors thank Stephanie C. Napier and Robert Whittier for kindly providing editorial assistance to the authors during the preparation of this manuscript.

Conflicts of interest

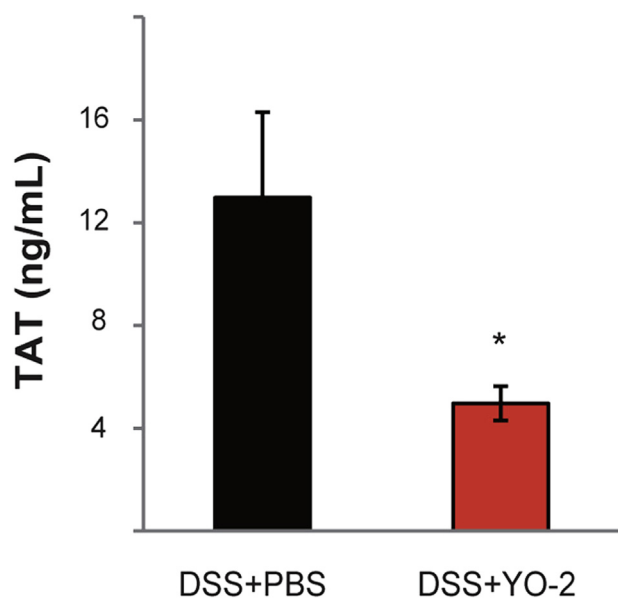
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Funding

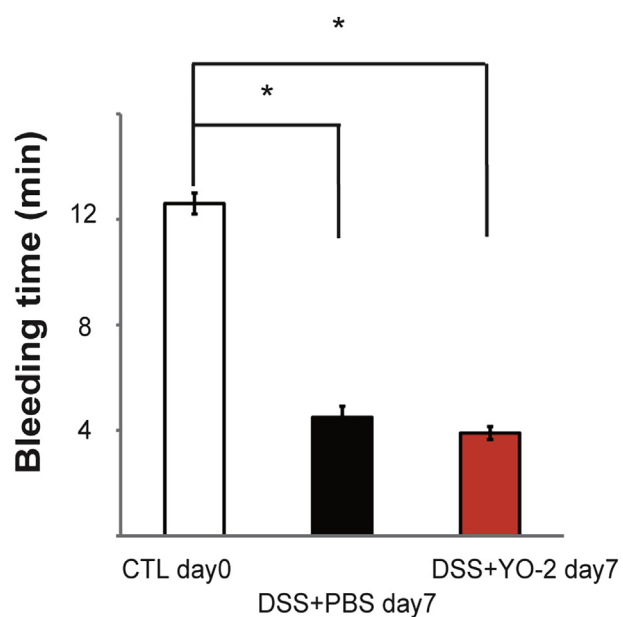
Supported by grants from the Japan Society for the Promotion of Science and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (K.H., B.H., Y.T.); Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (K.H.); the Mitsubishi Pharma Research Foundation (K.H.); Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology (B.H.); and the Program for Improvement of the Research Environment for Young Researchers (B.H.) funded by the Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology.

Supplementary Material

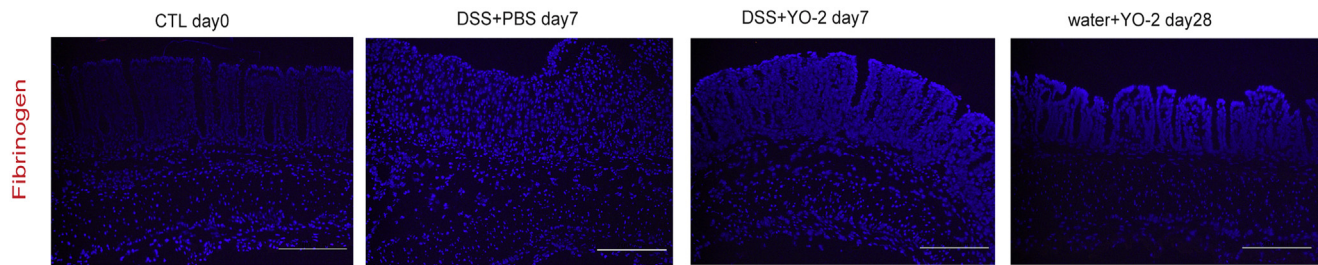
Supplementary Figures 1–9. DSS-induced mice were treated with or without YO-2. Data represent means \pm SEM. * $P < .05$, ** $P < .01$, and *** $P < .001$, determined by a 2-tailed Student t test.



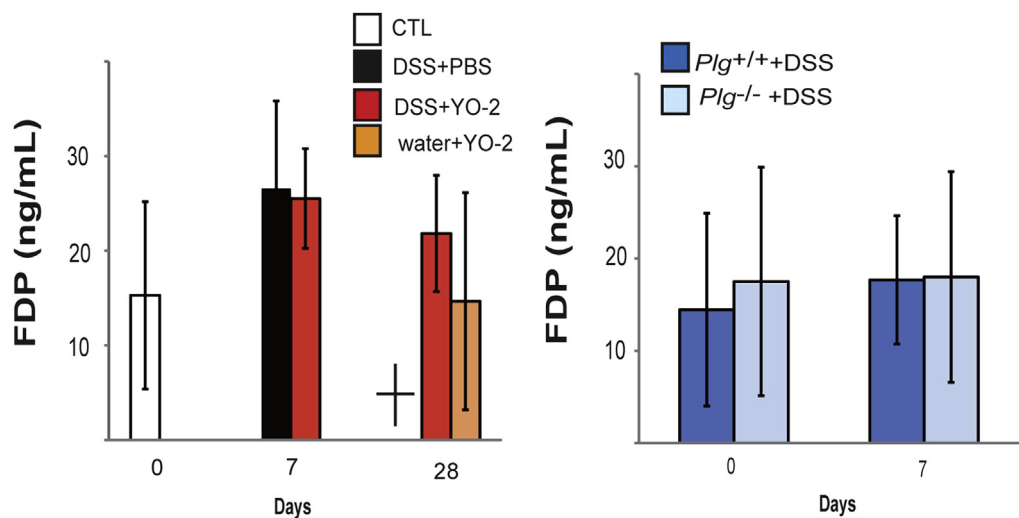
Supplementary Figure 1. The coagulation marker TAT is increased in the plasma of phosphate-buffered saline (PBS)-treated colitic mice, but not YO-2-treated mice, as determined by enzyme-linked immunosorbent assay at day 7. $n = 3/\text{group}$.



Supplementary Figure 2. Bleeding time was impaired in both phosphate-buffered saline (PBS)- and YO-2-treated DSS-induced mice at day 7. $n = 6/\text{group}$.



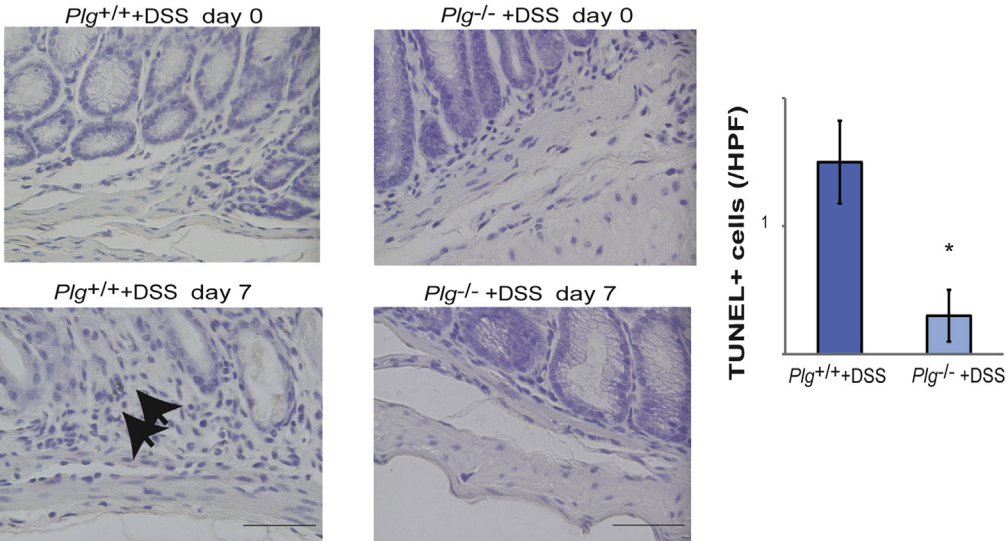
Supplementary Figure 3. Fibrin/fibrinogen immunostaining of colon tissues. No fibrin deposit was detected in colitic tissues after 7 days of DSS ingestion with/without YO-2 and in colon tissues of mice that had been treated for 28 days with YO-2. N = 3/group. CTL, control day 0; PBS, phosphate-buffered saline.



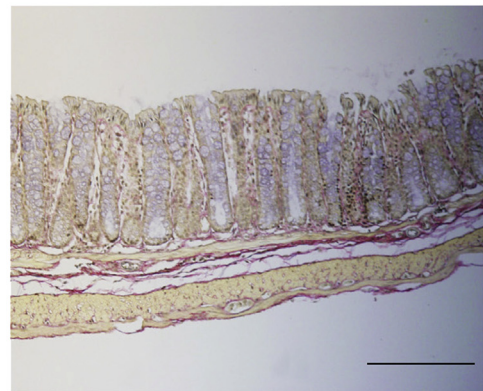
Supplementary Figure 4. FDP levels in blood samples of mice were analyzed. n = 3–6/group. CTL, control day 0; PBS, phosphate-buffered saline.

Supplementary

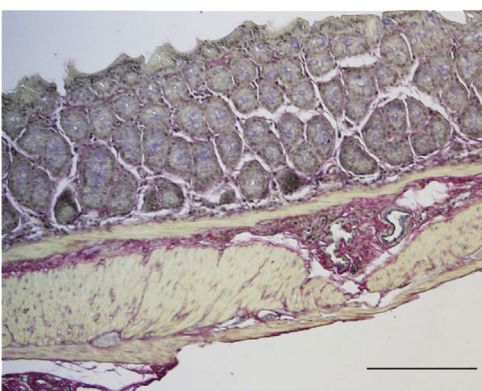
Figure 5. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of colon tissues showed an increased number of TUNEL⁺ cells in the control treated group (left panel). Arrows indicate TUNEL positively stained cells. *n* = 3/group. Right: Quantification of the number of TUNEL⁺ cells per high-power field (HPF). Scale bars: 20 μ m.



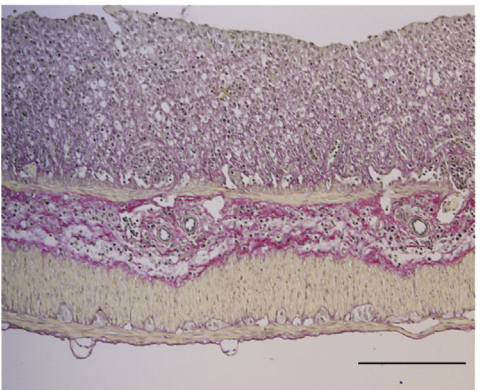
Plg^{+/+}+DSS day 0



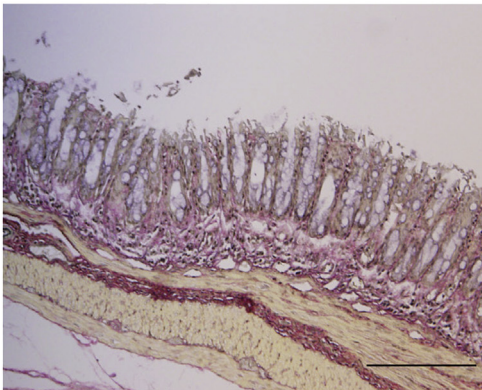
Plg^{-/-} +DSS day 0



Plg^{+/+}+DSS day 7

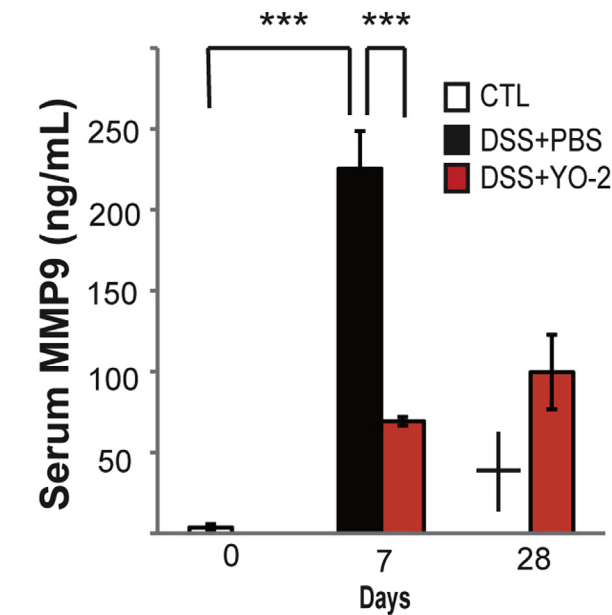


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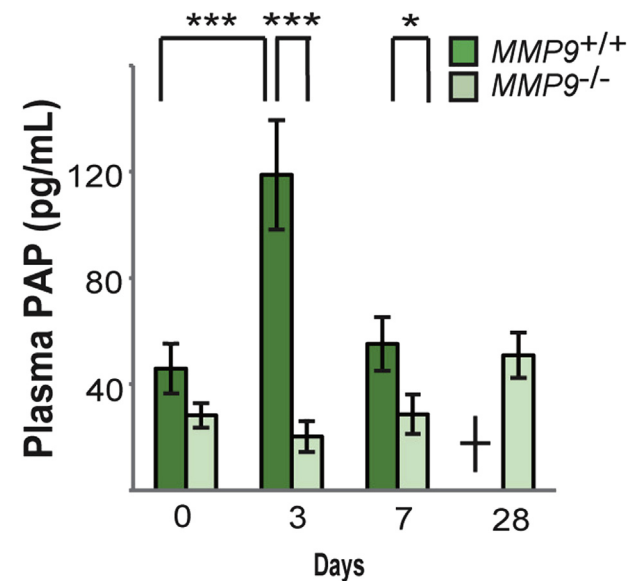


Supplementary

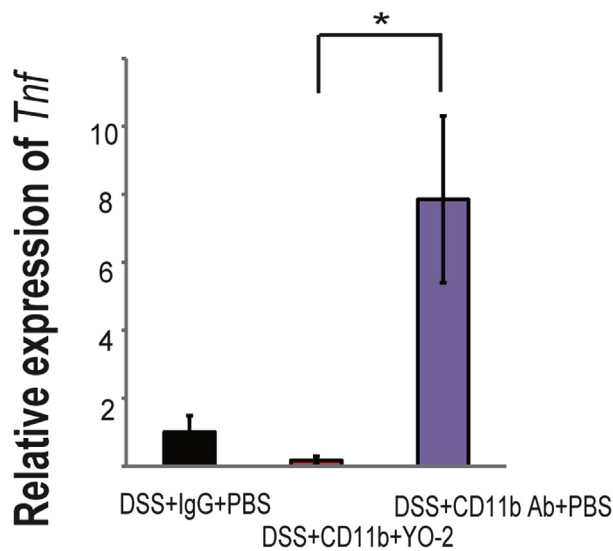
Figure 6. Elastica van Gieson staining (blue staining, used to differentiate between collagen and smooth muscle; collagen stains bright red; and cytoplasm, muscle, and fibrin stain yellow) of colon tissues obtained from DSS-treated Plg^{+/+} and Plg^{-/-} mice. Scale bars: 200 μ m.



Supplementary Figure 7. MMP9 serum levels assayed in YO-2- or phosphate-buffered saline (PBS)-treated C57BL/6 mice by enzyme-linked immunosorbent assay. $n = 3/\text{group}$.



Supplementary Figure 8. PAP plasma levels in DSS-treated *Mmp9*^{+/+} and *Mmp9*^{-/-} mice by enzyme-linked immunosorbent assay. $n = 3/\text{group}$.



Supplementary Figure 9. *Tnf* expression in circulating mononuclear cells of indicated groups at day 7. $n = 3/\text{group}$. PBS, phosphate-buffered saline.