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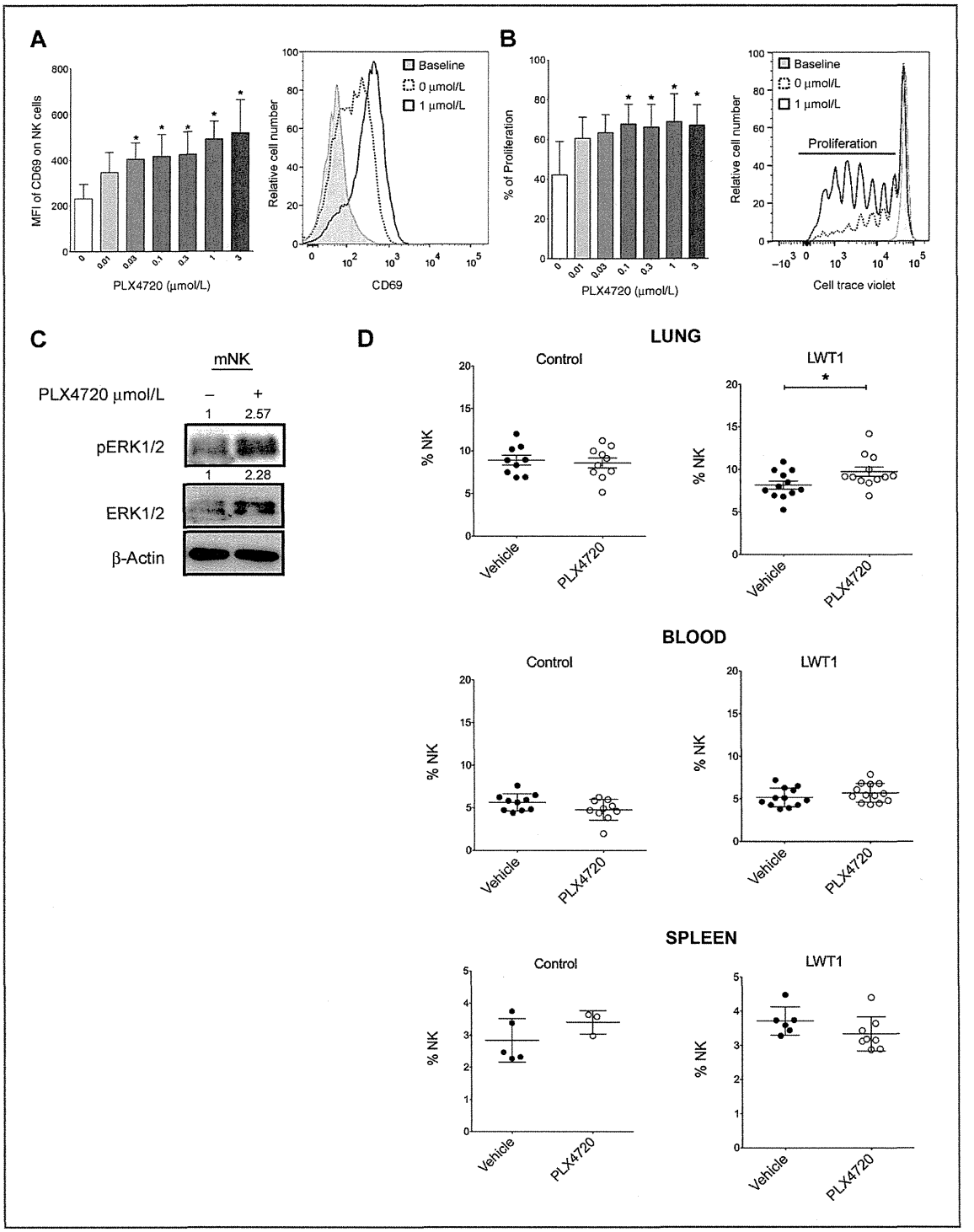

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or DMSO for 24 hours. We found that PLX4720 pretreatment of these target cells neither affected the NK cell-mediated killing of BRAF$^{V600E}$ nor BRAF$^{wt}$ tumor cells (Fig. 3E). Consistent with these findings, the expression levels of Rae-1, CD155, and MHC class I molecules were not affected by BRAF inhibition (Fig. 3F).

### PLX4720 directly impacts mouse NK cells

We next tested freshly purified NK cells activated with IL2 in the presence of increasing concentrations of PLX4720. Supplementation with PLX4720 significantly increased NK cell CD69 (Fig. 4A) and NK cell proliferation (Fig. 4B). In contrast, we did not observe any impact of PLX4720 on IFNγ production by NK cells in response to IL12 and IL18 cytokine combinations (Supplementary Fig. S3) or PLX4720 on NK cell-mediated cytotoxicity induced by IL15 (Supplementary Fig. S4). PLX4720 has been shown to increase ERK1/2 phosphorylation in BRAF$^{wt}$ cells (21, 22). We observed an increase in ERK1/2 phosphorylation after 24-hour PLX4720 treatment and an increase in total ERK1/2 expression after PLX4720 treatment, while β-actin levels were not modulated (Fig. 4C). PLX4720 also displayed NK cell modulatory functions *in vivo* because C57BL/6 mice bearing LWT1 lung metastases and treated with PLX4720 (20 mg/kg daily) for 24 hours had an increased NK cell frequency in the lungs compared with DMSO-treated mice or mice that had not been inoculated intravenously with LWT1 melanoma (Fig. 4D).

### PLX4720 and IL2 combination therapy suppresses melanoma metastasis

Given the demonstrated effects of PLX4720 and IL2 on NK cell proliferation *ex vivo* and the important role of NK cells in PLX4720 mechanism of action *in vivo*, we next assessed whether a combination of PLX4720 and IL2 could combine to suppress LWT1 metastasis in mice. We have previously shown the antitumor activity of IL2 in other models of tumor metastasis in mice (25) and herein used a similar dose/regimen of daily IL2 for 5 days concurrent with PLX4720 treatment. Notably, mice receiving a combination of PLX4720 and IL2 displayed significantly lower levels of LWT1 pulmonary metastasis compared with mice treated with either agent alone or DMSO/PBS (Fig. 5). Because IL2 has been standardly used in the treatment of advanced human malignant melanoma (26), these data suggest that this combination should now be further explored in this preclinical model.

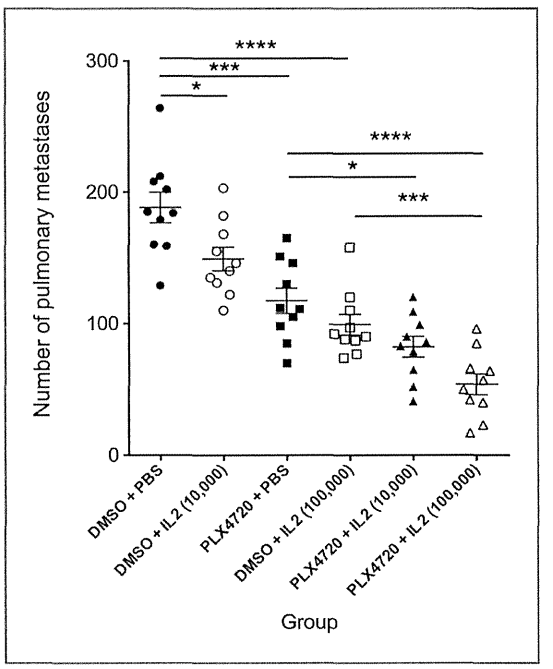


Figure 5. IL2 enhances the antimetastatic activity of PLX4720. A total of $5 \times 10^5$ LWT1 melanoma cells were injected i.v. into C57BL/6 WT mice and mice were subsequently treated with 20 mg/kg or equivalent amount of DMSO daily on days 1 to 7 and/or PBS or the indicated amount of IL2 (10,000 U or 100,000 U i.p.) given daily on days 1 to 5. Mice were sacrificed on day 14 and lungs harvested and fixed as described in Materials and Methods. Data are from two pooled experiments, with graphs representing the mean ± SEM pulmonary colonies of each group of five mice. Statistical analysis by Mann–Whitney test (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$).

### PLX4720 and IL2 increase human NK cell functions

Finally, to determine whether a similar mechanism may occur in PLX4720-treated cancer patients, we investigated whether PLX4720 directly impacted human NK cells. We observed a clear increase in ERK1/2 phosphorylation after PLX4720 treatment for 24 hours (Fig. 6A). Interestingly, like in mouse NK cells, an increase in total ERK1/2 was associated with PLX4720 treatment, whereas β-actin expression remained comparable between the two groups of NK cells (Fig. 6A). We next cultured NK cells for 6 days with IL2 and

Figure 4. PLX4720 enhances NK cell activation and proliferation. A and B, Cell Trace Violet-labeled purified NK cells were cultured with the indicated doses of PLX4720 in media supplemented with 300 U/mL IL2. Analysis of CD69 and proliferation was performed by flow cytometry. A, the mean ± SD CD69 expression of triplicates (left) and representative histograms showing CD69 expression (right) are shown. B, the mean ± SD percentage of divided NK cells (left) and representative histograms showing Cell Trace Violet dilution (right) after 3 days in culture are shown. A and B, data are representative of three independent experiments. Statistical analysis performed by unpaired Student *t* test, *, $P < 0.05$. C, $2 \times 10^6$ purified NK cells were cultured for 24 hours in the presence of PLX4720 or DMSO in media supplemented with 300 U/mL IL2. The phosphorylation of ERK1/2 and the total amount of ERK1/2 and β-actin were measured in cell lysate by Western blot analysis. Fold increase relative to no PLX4720 control is recorded above the lane. Representative images of three independent experiments are shown. D, naïve C57BL/6 WT mice (control) or C57BL/6 WT mice injected i.v. with $5 \times 10^5$ LWT1 melanoma cells (LWT1) were treated i.p. with 20 mg/kg PLX4720 or an equivalent amount of DMSO (vehicle) for 24 hours. Twenty-four hours after this treatment, mice were sacrificed and frequencies of NK cells in the lungs, peripheral blood, and spleens were determined by flow cytometry. The mean ± SEM percentages of NK cells are shown. Data pooled from two independent experiments are shown. *, $P < 0.05$ Mann–Whitney test.

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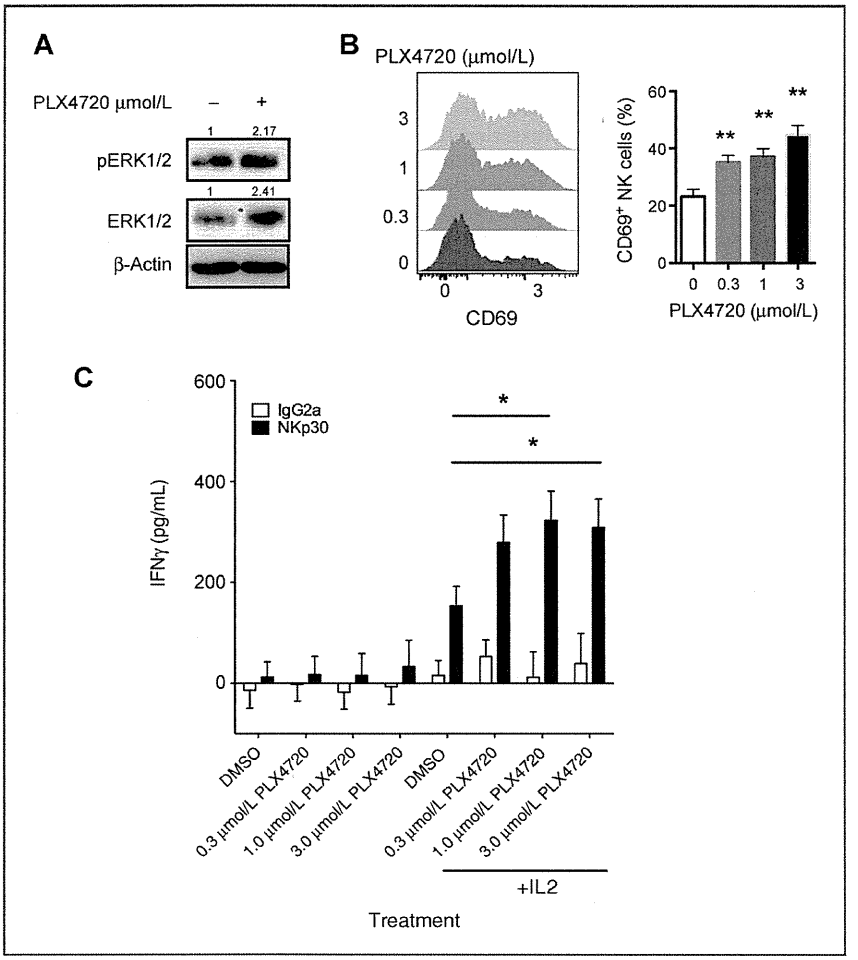


Figure 6. PLX4720 enhances human NK cell activation. A, 2 × $10^6$ human NK cells purified from PBMC of healthy donors were cultured for 24 hours in the presence of PLX4720 or DMSO in media supplemented with 300 U/mL IL2. The phosphorylation of ERK1/2, the total amount of ERK1/2 and β-actin were measured in cell lysate by Western blot analysis. Fold increase relative to no PLX4720 control is recorded above the lane. Representative images of two independent experiments are shown. B, purified human NK cells were cultured with the indicated doses of PLX4720 in media supplemented with 300 U/mL IL2. The representative histograms showing CD69 expression (left) and the mean ± SD CD69 expression of experimental replicates (right) are shown. Data are representative of four independent healthy donors. Statistical analysis was performed by unpaired Student *t* test, **, $P < 0.01$. C, purified human NK cells from nine donors were cultured in DMSO or PLX4720 (0.3–3.0 μmol/L as indicated) with or without IL2 (300 U/mL) for 20 hours. NK cells were then plated (5 × $10^4$/well) in wells coated with anti-NKp30 (±IL2 1,000 U/mL) and incubated for an additional 20 hours. Supernatants were harvested and IFNγ released in the supernatant measured by ELISA. Statistical analysis performed by Mann–Whitney test as indicated (*, $P < 0.05$).

increasing concentrations of PLX4720. We observed that PLX4720 induced a dose-dependent increase in CD69 expression on NK cells, demonstrating that PLX4720 potentiates human NK cell activation induced by IL2 (Fig. 6B). Finally, human NK cells secrete IFNγ when ligated via NKp30 in the context of IL2 activation (27). We assessed the effect of PLX4720 on NKp30/IL2-induced IFNγ secretion by purified NK cells from 9 different human donors. We observed that increasing concentrations of PLX (0.3 to 3 μmol/L) significantly enhanced the level of IFNγ above IL2 or NKp30 triggering alone (Fig. 6C). In contrast, PLX was unable to enhance the IFNγ secreted from human NK cells exposed to IL12/IL18 (data not shown).

## Discussion

Because of the lack of a mouse model, the mechanisms involved in the control of metastatic melanoma by $BRAF^{V600E}$ inhibitor has remained until now, unknown. In this study using a syngeneic $BRAF^{V600E}$ metastatic melanoma mouse model in immune-competent mice, we demonstrated that the antimetastatic effects of a selective $BRAF^{V600E}$ inhibitor PLX4720 require the action of host NK cells and perforin, and in part, the recognition/adhesion molecule, CD226. We showed that PLX4720 increases the phosphorylation of ERK1/2, CD69 expression, and proliferation of mouse NK cells *in vitro*. NK cell frequencies were significantly enhanced by PLX4720 specifically in the lungs of mice with $BRAF^{V600E}$ lung metastases. Furthermore, PLX4720 also increased human NK cell pERK1/2, CD69 expression, and IFNγ release in the context of anti-NKp30/IL2 stimulation. Finally, we demonstrated that therapy with a low or high dose of IL2 combined with PLX4720 was able to limit metastatic burden in mice. These findings revealed the importance of NK cells in treating $BRAF^{V600E}$ metastatic melanoma and now provide the basis for additionally exploring the curative potential of $BRAF^{V600E}$ inhibitors in combination with immunotherapies that engage NK cells.

Many reports have highlighted the importance of NK cells for immunotherapy, such as dendritic cell-based

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immunotherapy (28), IFNα and IL2 (29, 30). Now we can consider that BRAF$^{V600E}$ inhibitors may exert a large part of their antimetastatic activity via NK cells. Furthermore, the safety and efficacy of vemurafenib combined with IFN α-2b is currently under investigation in a new clinical trial (Clinical Trials.gov identifier NCT01943422), although the study is reportedly not recruiting yet. IFNα2b is an adjuvant treatment for patients with resected stage III melanoma, known to upregulate the expression of MHC class I on tumor cells, therefore increasing tumor antigenicity for recognition by CD8$^+$ T cells (31). Although engaging CD8$^+$ T cells is generally a positive approach to therapy, the recognition of MHC class I by NK cells triggers inhibitory signaling to reduce NK cell-mediated cytotoxicity and IFNγ production (23). Because here we found that the antimetastatic effects of PLX4720 require NK cells, it is possible that the upregulation of MHC class I on BRAF$^{V600E}$-mutant melanoma by IFNα2b might also impair the NK cell-related antimetastatic effects of the BRAF inhibitor, vemurafenib. The enhanced antimetastatic activity of low-dose IL2 and PLX4720 in this model of BRAF$^{V600E}$-mutant melanoma encourages us to explore other means to improve the NK cell-mediated control of metastasis in humans with BRAF$^{V600E}$-mutant melanoma. IL21 has shown promise in the treatment of malignant melanoma (32) and has antimetastatic activity and can terminally differentiate NK cells (33). Antibodies reactive with KIR, that relieve MHC class I inhibition, have also been shown to promote NK cell antitumor activity (34). Combinations of these agents with BRAF inhibitors in the treatment of melanoma are now worthy of further exploration.

Although cutaneous melanoma can be treated by surgical excision with great effectiveness when diagnosed early, metastasis of such tumors remains incurable (35). Therefore, investigation of strategies that eliminate remaining or resistant tumor cells is urgently needed. Once it was clear that NK cells were critical in controlling pulmonary metastasis of LWT1, we then tested whether NK cells were necessary for the antimetastatic activity of PLX4720. This was of important, because BRAF$^{V600E}$ inhibitors have mostly been examined in immune-deficient SCID mice that lack T and B cells, but do have NK cells (4). Here, we showed that the depletion of NK cells completely abolished the antimetastatic effects of PLX4720 treatment. We directly demonstrated *ex vivo* the enhancement of mouse NK cell pERK and CD69 expression, and proliferation by the BRAF$^{V600E}$ inhibitor in the context of IL2 culture. Human NK cell cultures revealed similar pERK1/2 and CD69 upregulation and an increase in IFNγ release by NK cells triggered via the activation receptor, NKp30, in the context of IL2. PLX4720 possibly increases ERK1/2 phosphorylation in BRAF WT cells (like NK cells) by a previously described mechanism (21, 22), whereas ATP-competitive inhibitors increase pERK1/2 levels by inhibiting CRAF inhibitory autophosphorylation, thereby activating the RAF and MAPK pathways.

Follow-up studies should now be performed on peripheral blood and other samples from human cancer patients receiving BRAF inhibitors to determine whether NK cell proliferation and effector functions are being regulated by treatment and how durable or transient these changes might be. It is possible that BRAF inhibitors may have a slightly different spectrum of activities on human NK cells to those observed in mouse NK cells and caution must be taken in translating observations from the mouse to humans. Furthermore, patients with melanoma are now receiving BRAF inhibitor and MEK inhibitor in combination and we might predict that this combination might abrogate any activation of BRAF WT NK cells. Thus far, the clinical focus has been to try and improve the curative potential of BRAF$^{V600E}$ inhibitors by combination with T-cell checkpoint inhibitors, such as anti-CTLA-4 or anti-PD-1. These combination approaches have surprisingly revealed some new toxicities (36). In contrast, therapies that attempt to improve the antimetastatic activities of BRAF$^{V600E}$ inhibitors by promoting NK cell function have not been rationally explored in the clinic. These approaches may improve overall survival in patients receiving BRAF inhibitors.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** L. Ferrari de Andrade, S.F. Ngiow, L. Martinet, M.J. Smyth
**Development of methodology:** L. Ferrari de Andrade, L. Martinet
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** L. Ferrari de Andrade, S.F. Ngiow, K. Stannard, S. Rusakiewicz, M. Kalimutho, S.-K. Tey, L. Zitvogel, L. Martinet, M.J. Smyth
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L. Ferrari de Andrade, S.F. Ngiow, S. Rusakiewicz, M.J. Smyth
**Writing, review, and/or revision of the manuscript:** L. Ferrari de Andrade, S.F. Ngiow, K.K. Khanna, L. Martinet, M.J. Smyth
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K. Takeda
**Study supervision:** S.F. Ngiow, K.K. Khanna, M.J. Smyth

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# Natural Killer Cells Are Essential for the Ability of BRAF Inhibitors to Control BRAF $^{V600E}$-Mutant Metastatic Melanoma

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# 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.

*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 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 $\alpha$ (TNF-$\alpha$) also is regulated via plasmin-mediated MMP activation. TNF-$\alpha$ 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]



*Authors share co-first authorship; §Authors share co-senior authorship.

***Abbreviations used in this paper:*** **Ab, antibody; CXCL5, C-X-C motif chemokine ligand 5; DAI, 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.**


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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 μL



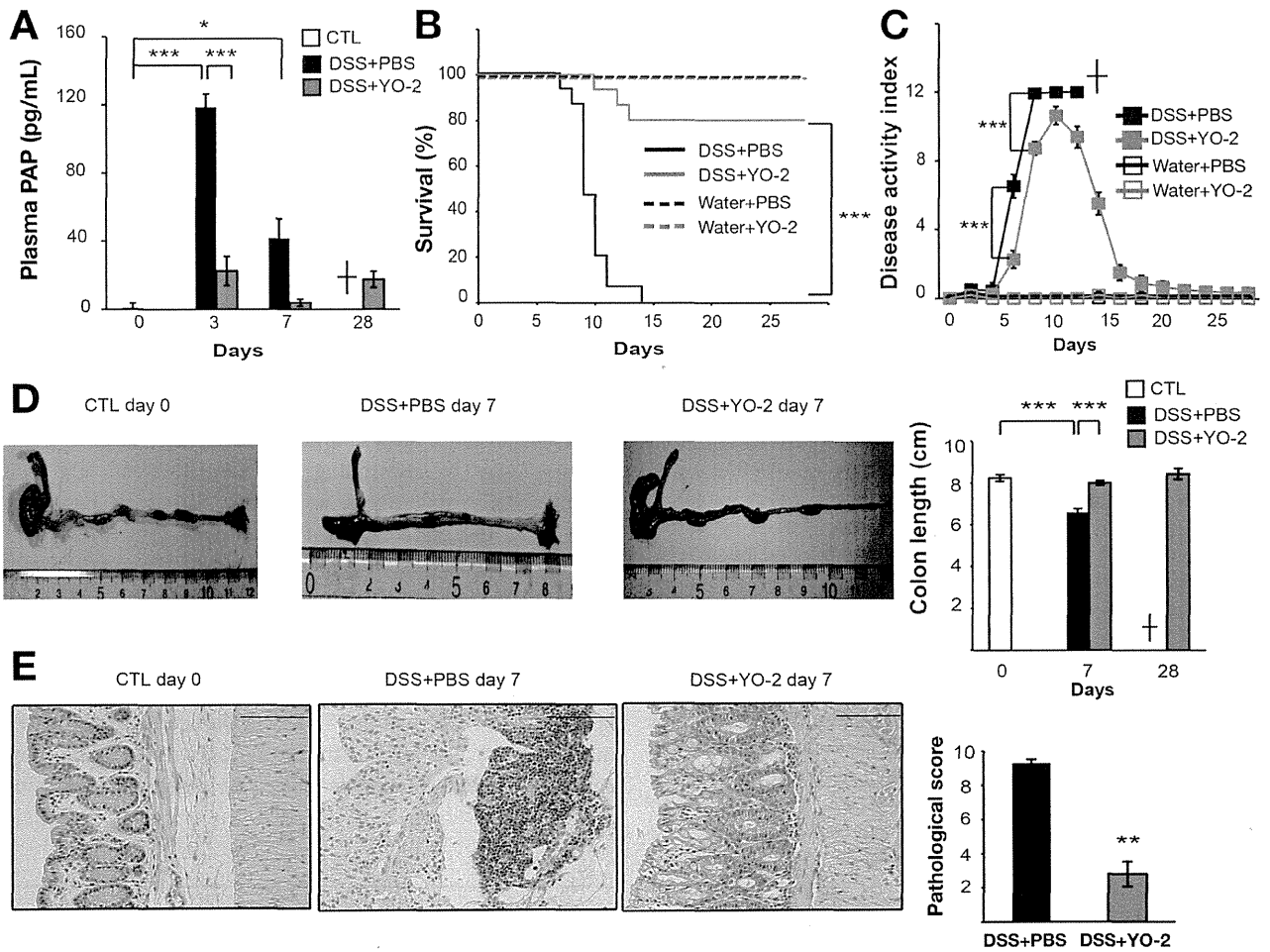



**Figure 1.** Plasmin inhibition prevents DSS-induced colitis progression. Colitis was induced by DSS and mice were injected with or without YO-2. (*A*) Plasma derived from mice treated with or without YO-2 was analyzed for PAP as a measure of active plasmin by enzyme-linked immunosorbent assay. n = 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 μm. Histologic inflammatory scores were determined for each section from DSS-induced mice treated with/without YO-2. n = 3/group. Values represent means ± 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 μg/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,

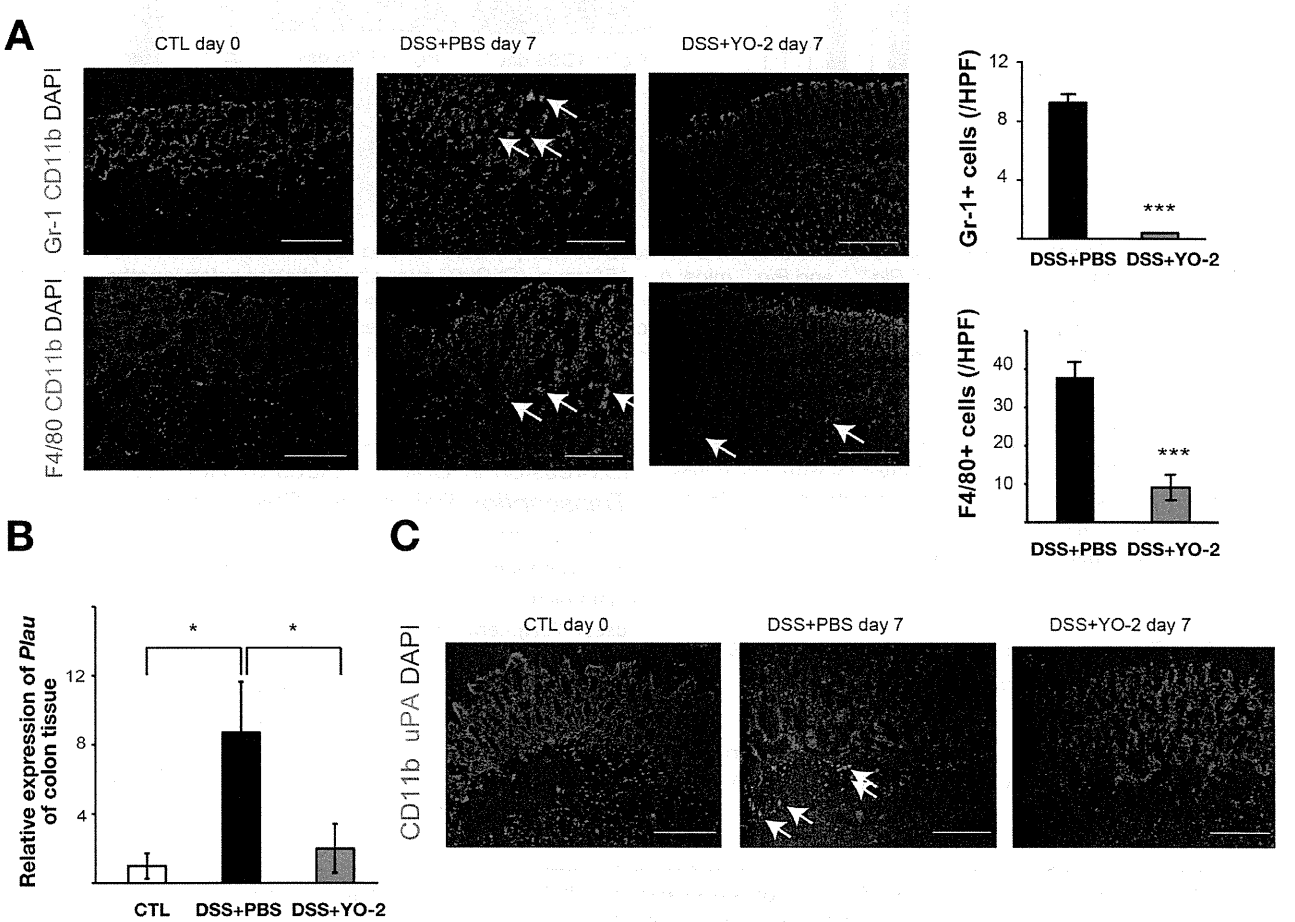



**Figure 2.** Plasmin inhibition suppresses the influx of myeloid cells into colonic tissue. (*A*) Representative immunohistochemical images of Gr-1/CD11b- and F4/80/CD11b-stained colon sections retrieved 7 days after DSS induction of C57BL/6 mice treated with or without YO-2. *Arrows* indicate positively stained cells. n = 3/group. *Scale bars*: 200 μm. *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 μm. Data represent means ± 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.

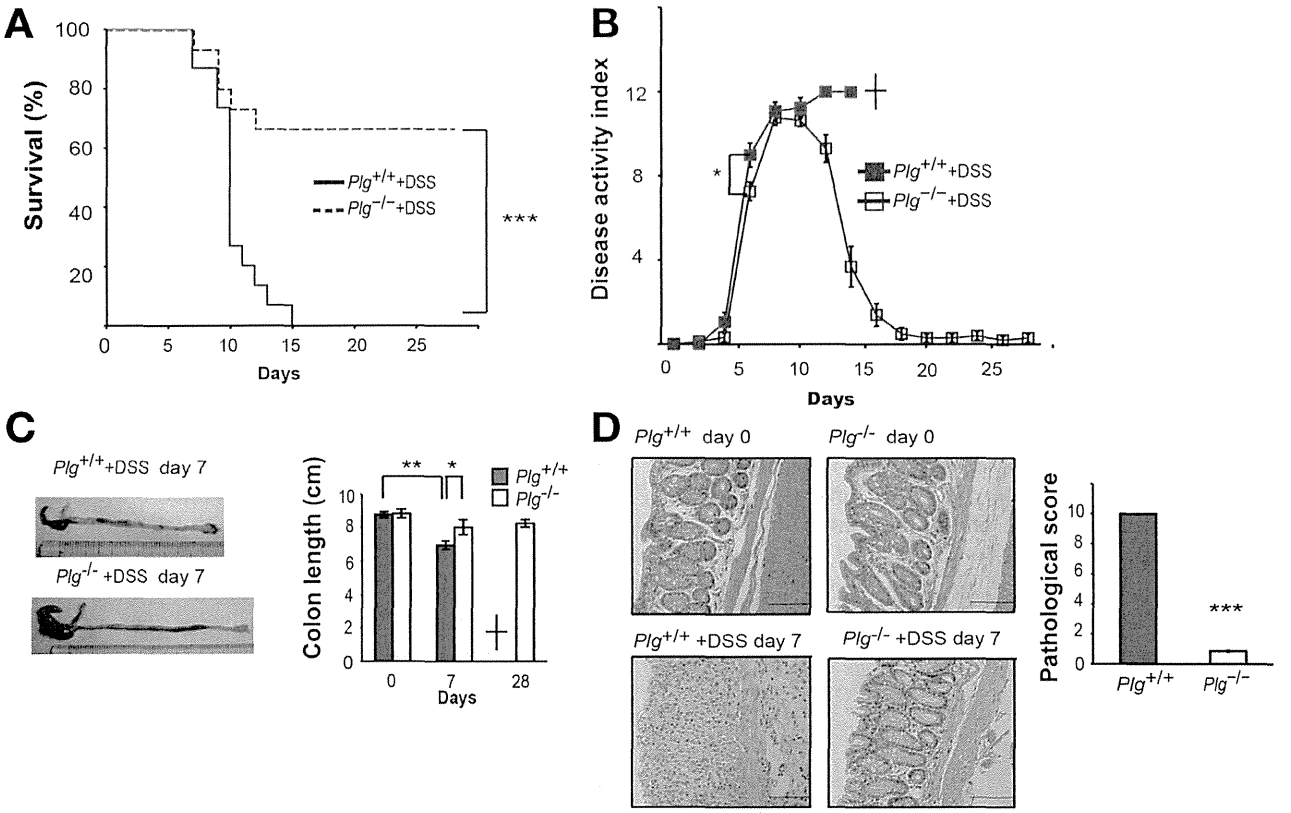



**Figure 3.** *Plg*-deficient mice are less susceptible to DSS-induced colitis. (*A*) Survival and (*B*) DAI were determined over the indicated time period in DSS-treated $Plg^{+/+}$ and $Plg^{-/-}$ mice: $n = 15$/group. (*C*) $Plg^{-/-}$ mice show shorter colon lengths. $n = 9$ for each group. *Right*: Representative H&E-stained colon sections from DSS-treated $Plg^{+/+}$ and $Plg^{-/-}$ mice. *Scale bars*: 200 μm. n = 3/group for all experiments. (*D*) *Right*: Pathologic scores obtained using colon tissues. Values represent means ± 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.

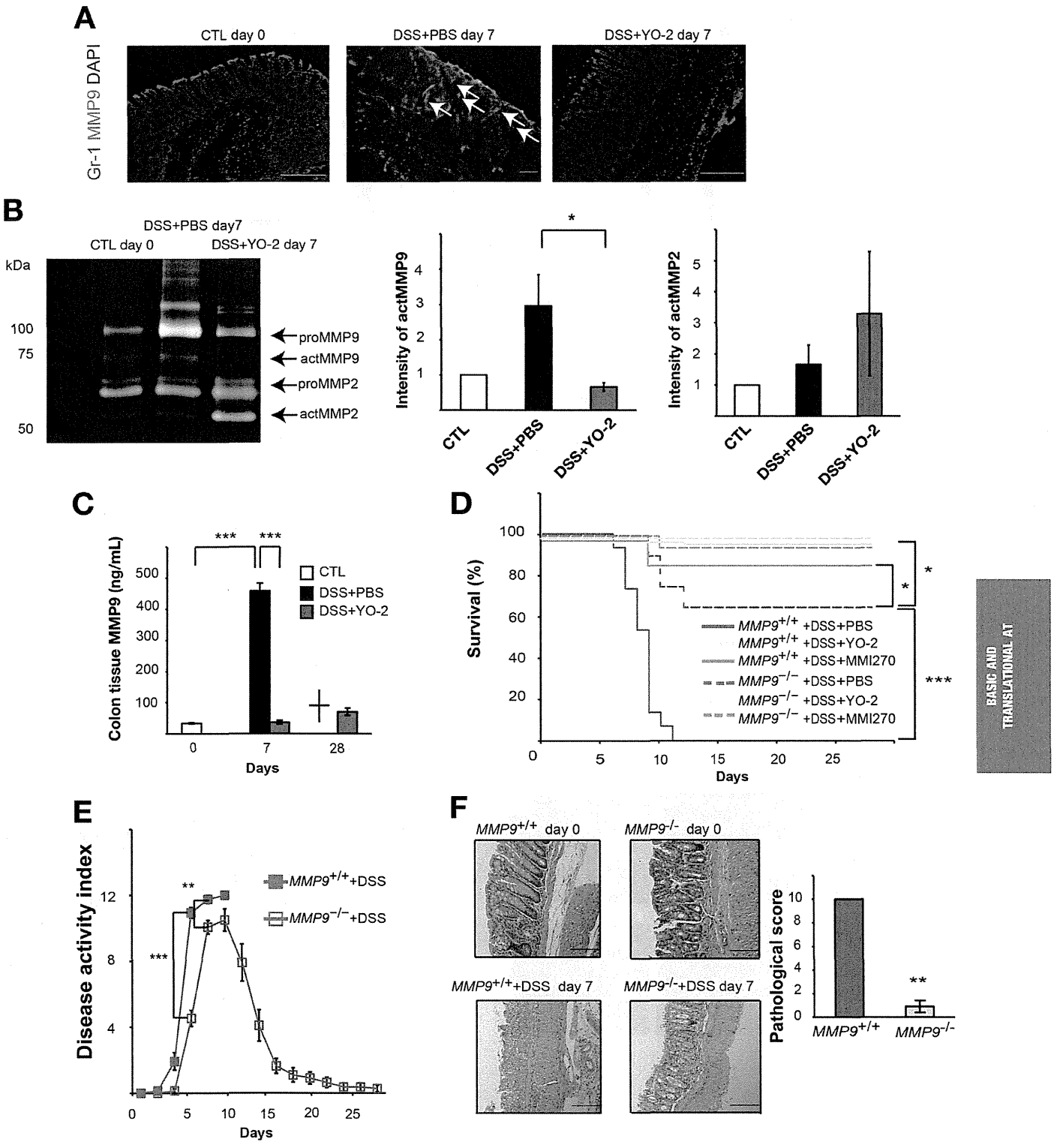
### *Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay*

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'-gtactgctttgtggact-3' and 5'-tgctgttggtaagttgtctg-3'; *Tnf*: 5'-gccgatttgctatctcatac-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 ± 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 μm. 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.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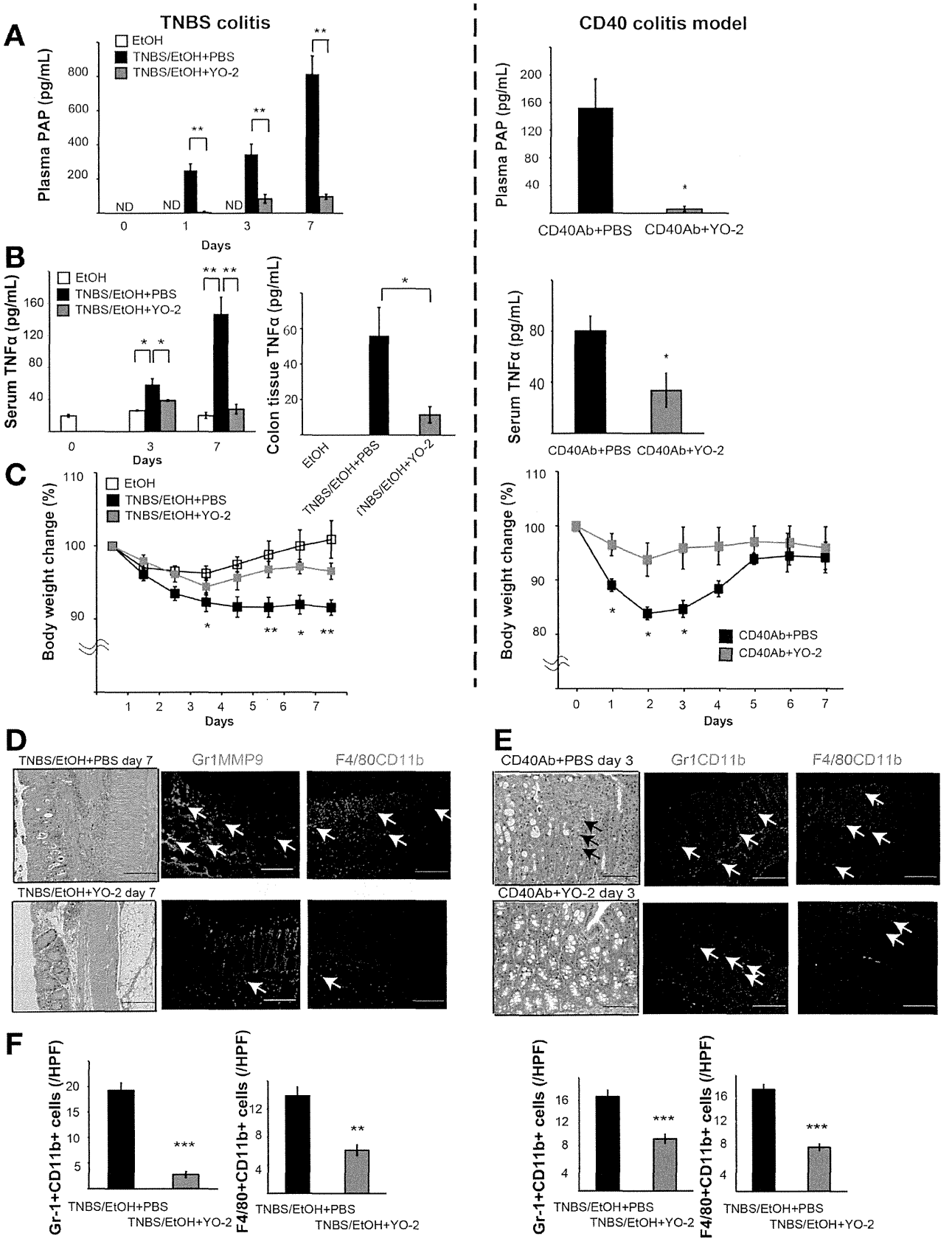


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5'-ggtatatgggctcataccag-3'; *Il1b*: 5'-gcaactgttcctgaactc-3' and 5'-ctcggagcctgtagtgca-3'; *Il6*: 5'-tgacaggatgcagaaggaga-3' and 5'-gctggaaggtggacagtgag-3'; *Cxcl5*: 5'-gcatttctgttgctgttcacgctg-3' and 5'-cctccttctggtttttcagtttagc-3'; *Actb* control: 5'-gtatgaa-caacgatgatg-3' and 5'-ccagaagaccagaggaaa -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-$\alpha$ (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$\beta$ and IL6 (Biolegend, San Diego, CA).

### Gelatin Zymography

Plasma samples were treated with 20 $\mu$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 ($1 \times 10^6$) were placed in 24-well Transwell inserts with 8-$\mu$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 ± 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 1*A*). 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 1*A*). 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

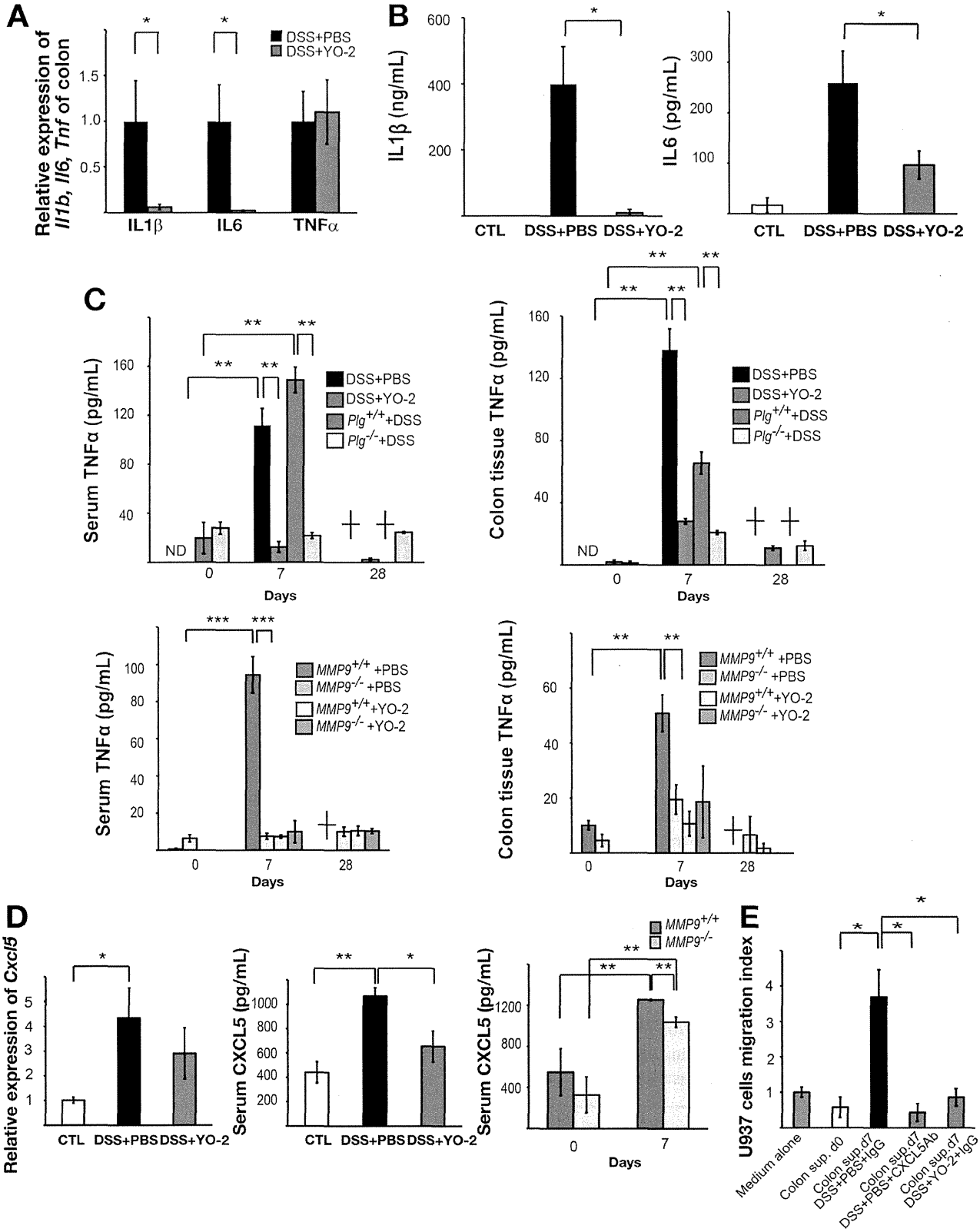
Plasmin inhibition by YO-2 treatment protected against DSS-induced colitis-associated lethality, and ameliorated the high DAI (a numeric value reflecting weight loss, diarrhea, and bleeding), and prevented shortening of the colon (Figure 1*B–D*). In line with these observations, colon sections of YO-2–treated mice showed less severe mucosal damage, loss of goblet cells, and inflammatory cell infiltration (Figure 1*E*), 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 2*A*). *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-$\alpha$ 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 $\mu$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 $\mu$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 ± SEM. **P* < .05, ***P* < .01, and ****P* < .001, determined by a 2-tailed Student *t* test. HPF, high-power field.

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(Figure 2*B*), 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 2*C*). 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 3*A*), a lower DAI (Figure 3*B*), and diminished colon length shortening (Figure 3*B* 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 3*D*). 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

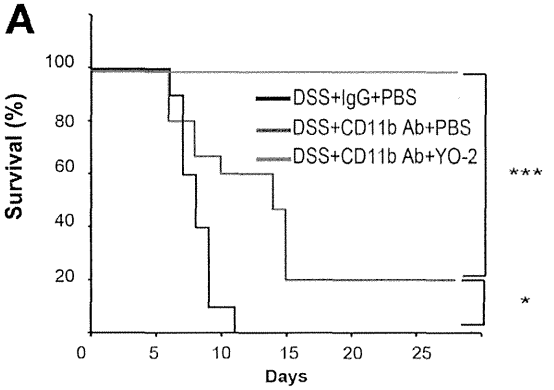
Because plasmin can activate MMPs, and MMPs such as MMP3, MMP7, and MMP12 are linked to the progression of colitis,[25] we hypothesized that plasmin, by activating proteases such as MMP9, might contribute to colitis progression in vivo. Immunoreactive MMP9 was observed in epithelial cells and neutrophils of colon sections derived from DSS-induced colitic mice, but not in YO-2–treated mice (Figure 4*A*). 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 4*B*). Similarly, colon supernatants of YO-2–treated DSS-induced colitic mice contained less total MMP9 (Figure 4*C*). DSS-induced colitic *Mmp9*$^{-/-}$ mice showed an improved survival rate, a lower DAI, prevented infiltration of inflammatory cells, and colonic tissue destruction (Figure 4*D–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 4*D*). 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-$\alpha$ level was observed (Figure 5*A* and *B*). YO-2 treatment prevented colitis-related body weight loss (Figure 5*C*) and the destruction of the colonic epithelium in both colitis models (Figure 5*D* 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 5*D*). In addition, YO-2 treatment reduced the number of infiltrating inflammatory Gr-1$^+$ and F4/80$^+$ myeloid cells (Figure 5*F*). 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$\beta$ 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-$\alpha$ 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 ± SEM. **P* < .05 and ***P* < .01, determined by a 2-tailed Student *t* test. CTL, control day 0.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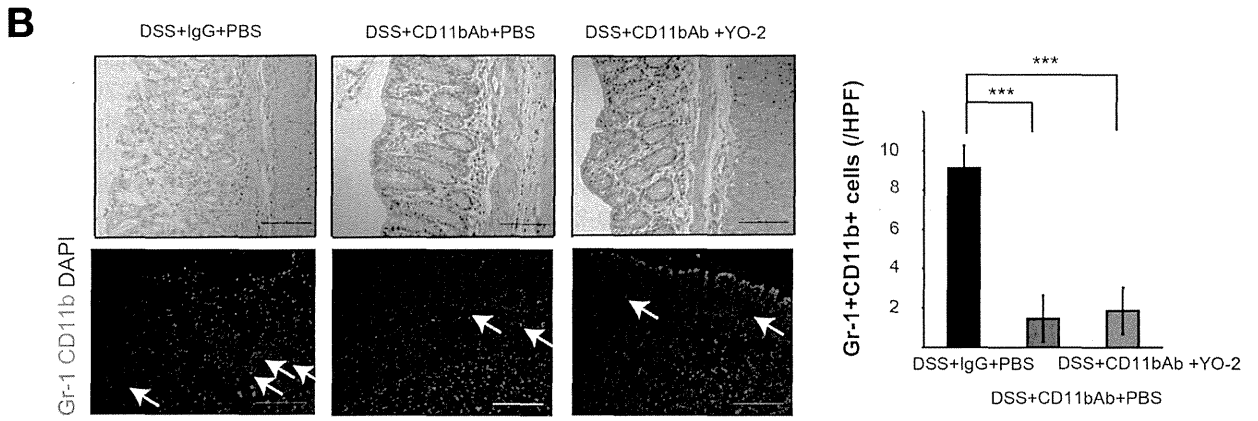
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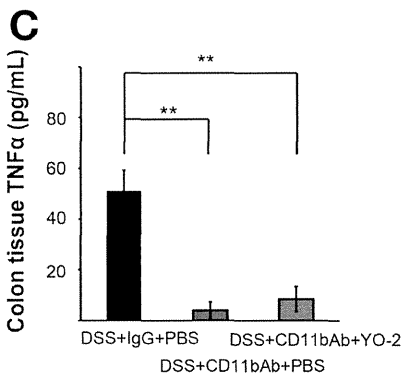
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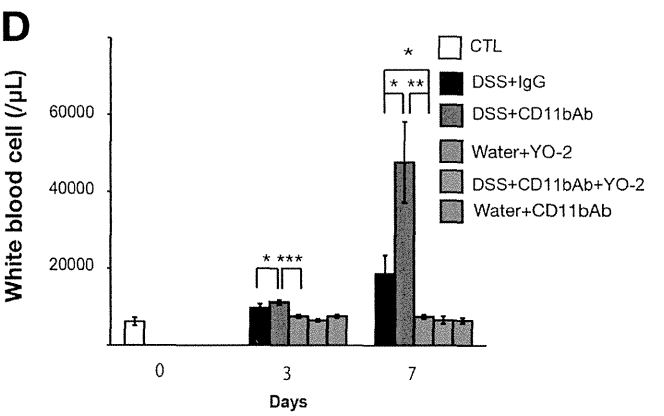
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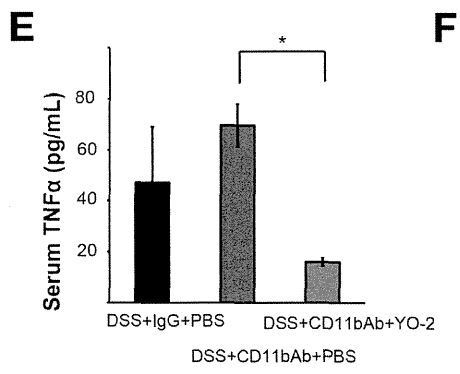
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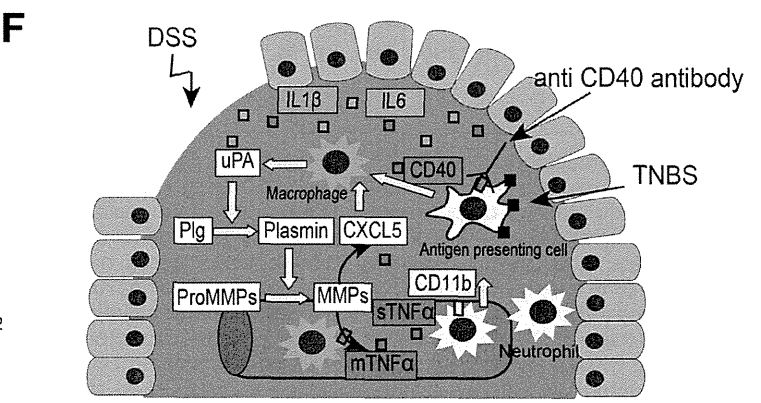
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ProMMPs
MMPs
sTNFα
mTNFα
Neutrophil

### *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 6*A*) in colonic extracts and circulating IL1$\beta$ and IL6 (Figure 6*B*) was lower in YO-2–treated colitic mice. Plasmin deletion or inhibition and *Mmp9* deficiency prevented the increase in circulating TNF-$\alpha$ serum levels and suppressed TNF-$\alpha$ release from colonic supernatants after DSS induction (Figure 6*A–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 6*D*). 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 6*E*). 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 7*A*). Similar to previous reports,[28] 80% of DSS-induced mice treated with neutralizing Abs against CD11b died (Figure 7*A*), although CD11b$^+$ cell influx into colonic tissues (Figure 7*B*) and local colonic TNF-$\alpha$ production was blocked (Figure 7*C*). 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-$\alpha$ increase correlated with leukocytosis by mononuclear cells, whereas local colonic TNF-$\alpha$ production correlated with the number of infiltrated CD11b$^+$ cells (Figure 7*C–E*). Survival, white blood cell counts, histopathologic changes, and TNF-$\alpha$ serum level increase were suppressed after co-injection of YO-2 with CD11b Abs (Figure 7*A–E*). Our data suggest that plasmin promotes colitis progression by accelerating leukocytosis and leukocyte activation, resulting in the release of TNF-$\alpha$, 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-$\alpha$ 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-$\alpha$ 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 $\mu$m. (*C*) TNF-$\alpha$ 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-$\alpha$ protein was determined in plasma samples taken at day 7. Dates represent means ± 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-$\alpha$) 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-$\alpha$ 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 7*F*.

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-$\alpha$ release both systemically and locally in the colonic environment. TNF-$\alpha$ release involves a process called shedding, which involves the protease ADAM17/TNF-$\alpha$ converting enzyme, but also other MMPs such as MMP1, MMP7, and MMP9.[36] Our data indicate that plasmin-dependent TNF-$\alpha$ production requires endogenous MMP9, which promotes TNF-$\alpha$ 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-$\alpha$, 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$\beta$ 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-$\alpha$ 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$\beta$-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-$\alpha$ 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.

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