Inhibition of plasmin protects against experimental colitis by suppressing the matrix metalloproteinase-9-mediated cytokine release from myeloid cells

Shinya Munakata1,2,3,4, Yoshihiko Tashiro1,2,3,4, Chiemi Nishida1,3, Aki Sato1, Hiromitsu Komiyama1,2, Hiroshi Shimazu1, Douaa Dhahri3, Yousef Salama3, Salita Eiamboonsert3, Kazuyoshi Takeda5, Hideo Yagita5, Yuko Tsuda6, Yoshio Okada6, Hiromitsu Nakauchi1, Kazuhiro Sakamoto2, Beate Heissig1,3,7,8 and Koichi Hattori1,7,8

1 Stem Cell Regulation, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
2 Department of Coloproctological Surgery, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
3 Stem Cell Dynamics, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
5 Department of Immunology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
6 Faculty of Pharmaceutical Sciences, Kobe Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe 651-2180, Japan
7 Atopy (Allergy) Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

4 Author names in bold designate shared co-first authorship
8 share senior authorship

Address correspondence to:
Koichi Hattori, MD, PhD
Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, JAPAN.
Phone: +81-3-5449-5136,
Fax: +81-3-5449-5742,
E-mail: khattori@ims.u-tokyo.ac.jp

Short title: Plasmin drives colitis through myeloid cell influx
Munakata et al,

**Author contributions:**
Study concept and design (SM, YT, KS, BH, KH); data acquisition (SM, YT, HK, HS, AS, CN, YS, DD, SE, BH, KH); analysis and interpretation of data (SM, YT, HS, AS, CN, BH, KH); drafting of the manuscript (BH, SM, KH); critical revision of the manuscript (KH); obtained funding (KH, BH).

**No conflicts of interest exist.**

**Grant support:** This work was 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 (MEXT) (K.H.; B.H.); Grant-in-Aid for Scientific Research on Priority Areas from the MEXT (K.H.), Mitsubishi Pharma Research Foundation (K.H), Grant-in-Aid for Scientific Research on Innovative Areas from the MEXT (B.H.), 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 MEXT, Japan.

**Abbreviations:** plasminogen, Plg; plasmin, Plm; dextran sulfate sodium, DSS; inflammatory bowel disease, IBD; tumor necrosis factor, TNF; trinitrobenzene sulfonic acid, TNBS; phosphate-buffered saline, PBS; matrix metalloproteinase, MMP; interleukin, IL; tissue-type plasminogen activator, tPA; urokinase-type plasminogen activator, uPA; antibody, Ab; C-X-C motif chemokine 5, CXCL5
Munakata et al,

**Acknowledgements:** We thank Stephanie C. Napier and Robert Whittier for kindly providing editorial assistance to the authors during the preparation of this manuscript.
ABSTRACT (260 words)  
BACKGROUND & AIMS: The activation of proteases, like plasmin or matrix metalloproteinases (MMPs), has been reported in patients during the active phase of inflammatory bowel diseases. Here, we investigated the effect of plasmin on acute colitis progression.

METHODS: Mucosal or blood samples were taken from mice with colitis induced by the administration of dextran sulfate sodium (DSS), trinitrobenzene sulfonic acid (TNBS) or CD40 antibody. The effect of pharmacological plasmin inhibition by YO-2, blocking the active site of plasmin or gene deficiency in plasminogen/plasmin or MMP-9 on mucosal inflammation was assessed. Furthermore, the effect of plasmin to control the chemo-/cytokine response and leukocyte recruitment during colitis progression was examined.

RESULTS: Circulating plasmin was increased in mice with experimental colitis. Pharmacological and genetic plasmin inhibition prevented colitis progression by reducing the infiltration of Gr1⁺ neutrophils and F4/80⁺ macrophages into colonic tissues and by reducing the production of colonic inflammatory cytokines and chemokines. This process required the activation of endogenous MMP-9 as shown using MMP-9⁻/⁻ mice. Plasmin inhibitor-treated and Plg⁻/⁻ or MMP-9⁻/⁻ mice showed decreased TNF-α and CXCL5 serum levels.

CONCLUSIONS: Our results indicate that plasmin induces a vicious cycle whereby activation of the fibrinolytic system occurs in the early phase of colitis onset, activating MMPs like MMP-9 or others, and providing a proteolytic environment that stimulates myeloid cell influx into colon epithelium and production of TNF-α and CXCL5. In turn, myeloid CD11b⁺ cells release the urokinase plasminogen activator further accelerating plasmin production. Thus, disruption of the plasmin-driven chronic inflammatory circuit is a promising strategy for therapeutic intervention in colitis.

Key Words: matrix metalloproteinase, plasmin, tumor necrosis factor, colitis, inflammatory bowel disease
Munakata et al,

**No conflicts of interest exist.**

**Grant support:** This work was 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 (MEXT) (K.H.; B.H.); Grant-in-Aid for Scientific Research on Priority Areas from the MEXT (K.H.), The Novartis Foundation for the Promotion of Science (B.H), Mitsubishi Pharma Research Foundation (K.H), grant from the Novartis Foundation for the promotion of Science (B.H.), Grant-in-Aid for Scientific Research on Innovative Areas from the MEXT (B.H.), 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 MEXT, Japan.

**Acknowledgements:** We thank Stephanie C. Napier and Whittier Robert for kindly providing editorial assistance to the authors during the preparation of this manuscript (no funding source was used).
Introduction

Crohn’s disease (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel disease (IBD) in humans, result from the interaction of genetic and environmental factors that ultimately promote an immunopathologic process leading to chronic inflammation. Treatment of chronic IBD generally relieves symptoms, but is not curative. Activation of proteases like matrix metalloproteinases (MMPs), or serine proteases, like plasmin, can break down the intestinal epithelial barrier due to their potential to degrade components of the extracellular matrix, resulting in the invasion of inflammatory cells.

MMP-9 expressed by epithelial cells plays an important role in the development of colitis by modulating cell-matrix interaction and wound healing. MMP inhibitor treatment is effective for colitis, but patients described side effects including severe lethargy and rigors. Therefore, the therapeutic effects of more selective inhibitors of disease-associated MMPs are currently under investigation. Another way of controlling the activation of MMPs is through the serine protease plasmin, which has been shown to be important for the release of cytokines/chemokines like Kit ligand, monocyte chemoattractant protein-1, chemokine (C-X-C motif) receptor 5 and basic fibroblastic growth factor. It is conceivable that the production of proinflammatory cytokines like tumor necrosis factor-α (TNF-α) is also regulated via
Munakata et al,

plasmin-mediated MMP activation. TNF-α is rapidly induced in the intestinal mucosa upon initial activation of immune cells, and this induction is linked to disease progression during IBD.12

Plasmin, a key enzyme of the fibrinolytic cascade, can degrade the fibrin clot. It is generated by conversion from its precursor, plasminogen (Plg), by plasminogen activators (PA), tissue-type PA and urokinase-type PA (uPA). Early clinical studies demonstrated that circulating monocytes derived from chronic IBD patients showed increased secretion of PA13 and that disease activity depended upon upregulation of uPA in the active stage of the disease.14

In the present study, we examined the role of the fibrinolytic system in experimental models of colitis. We show that genetic and pharmacological plasmin inhibition prevents progression of IBD in experimental models of colitis, and ameliorates the disease in part through the MMP-9 inactivation-dependent reduction in the influx of inflammatory cells and production of inflammatory cytokines.

**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 dextran sulfate sodium (DSS) induced colitis as a model. An increase in
plasmin-antiplasmin complex (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 ELISA (Figure 1A). We next evaluated the effects of plasmin inhibitor YO-2 administration on fibrinolytic factor and coagulation factor release into circulation during colitis. The plasmin inhibitor YO-2 blocks the catalytic site of plasmin, and thereby efficiently inhibits active circulating plasmin.15 YO-2 treatment inhibited the systemic increase in PAP in plasma within the colon of DSS-treated animals (Figure 1A). Clinically, patients with colitis often show both excessive fibrinolysis and coagulation.16 As the function of plm is to dissolve fibrin clots, plm inhibition might alter coagulation and fibrin deposition/clot formation. However, YO-2 prevented the increase of the coagulation marker thrombin–antithrombin (TAT) on day 7 after colitis induction (Supplementary Figure 1). No obvious thrombus formation or bleeding signs were found in YO-2 treated mice. Bleeding time was shortened in both YO-2 treated and PBS-treated colitic mice due to increased coagulation (Supplementary Figure 2). No fibrin(ogen) staining pattern was observed in colon tissues of YO-2- and vehicle-treated colitic mice (Supplementary Figure 3). When plasmin breaks down fibrin, fibrin degradation products (FDPs) are produced. No significant change in plasma FDP levels was observed in blood samples of DSS-induced colitic mice treated with or
Munakata et al,

without YO-2 (Supplementary Figure 4). The data indicated that plasmin elevation occurs during the early phase of colitis, but no signs of fibrinolysis were found.

**Pharmacological 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 disease index activity (a numerical value reflecting weight loss, diarrhea and bleeding), and prevented the shortening of the colon (Figure 1B-D). In line with these observations, colon tissue 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 releasing myelomonocytic 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). Urokinase plasminogen activator (uPA) (Figure 2B), but not tissue type PA (data not shown) expression was upregulated in colon tissues of DSS-treated mice. In accordance with previous studies,\(^{17}\) YO-2 treatment prevented the influx of uPA-coexpressing CD11b\(^+\) cells in colonic tissues (Figure 2C). These data indicate that local
Munakata et al,

myelomonocytic cells are a source of uPA during the colitis process.

**Reduced disease severity in Plg^-/- mice with DSS-induced colitis**

To further study the role of plasminogen (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 disease activity index (Figure 3B), and a diminished colon length shortening (Figure 3B-C). Because colon shortening might be due to increased cell apoptosis, TUNEL staining on colon sections was used to determine apoptosis. Fewer TUNEL+ cells were observed in *Plg^-/-* mice, indicating that *Plg* deficiency protects from apoptosis in colitic mice (Supplementary Figure 5). *Plg^-/-* mice showed reduced histological signs of inflammation, with fewer inflammatory infiltrates and ulcerations (Figure 3D). Less deposition of collagen and elastic fiber as stained by Elastica van Gieson staining was observed in *Plg^-/-* colitic 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 MMP-9 dependent manner**
Munakata et al,

Because plasmin can activate MMPs, and MMPs like MMP-3, -7, and -12 are linked to the progression of colitis,\textsuperscript{18} we hypothesized that plasmin by preventing the activation of proteases like MMP-9 might contribute to colitis progression in vivo.

Immunoreactive MMP-9 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 rise in total plasma MMP-9 in YO-2 treated DSS-induced colitic mice (Supplementary Figure 7), with a decrease in both the pro-MMP and active form of MMP-9, but not MMP-2, as determined by zymography (Figure 4B). Similarly, colon supernatants of YO-2 treated DSS-induced colitic mice contained less MMP-9 total protein (Figure 4C). DSS-induced colitic \textit{MMP-9}^{-/-} mice showed improved survival, a lower disease activity index, reduced infiltration of inflammatory cells and colonic tissue destruction (Figure 4D-F). The broad-spectrum MMP inhibitor MMI270, which inhibits several MMPs, when compared to genetic MMP-9 deletion (MMP-9^{-/-}) alone ameliorated colitis progression (Figure 4D). But comparing YO-2 and MMI270 treated mice, YO-2 treated mice showed the best survival, which was due to the fact that 10\% of MMI270 treated mice died after suffering from a musculoskeletal syndrome, a known side effect of MMP inhibitors containing a hydroxamate structure.\textsuperscript{19} No side effects were observed in YO-2 treated mice.
Munakata et al,

**Plasmin inhibition ameliorates colitis after TNBS and CD40 induction**

We extended our analysis by evaluating the effect of plasmin inhibition in two other colitis models: the TNBS-induced colitis and the anti-CD40 colitis model. In YO-2 treated colitic mice, no increase in circulating plasmin and no augmentation in colon-derived or circulating TNF-α 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 MMP-9 was found in colonic tissue of PBS, 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 into colitis tissues (Figure 5F). These data suggest that plasmin inhibition suppressed clinical progression, colitis-associated cytokine storm and MMP-9 expression in three murine models of colitis (DSS- and TNBS- and CD40- induced).

**Plasmin and MMP-9 deficiency reduce the cytokine storm associated with colitis.** We next examined typical IBD-associated cytokines in DSS-induced colitic mice. The expression of IL-1β and IL-6 mRNA (Figure 6A) in colonic extracts and circulating IL-1β and IL-6 protein (Figure 6B) was lower in YO-2-treated DSS-induced colitic mice. Plasmin deletion or inhibition and MMP-9 deficiency prevented the increase in
Munakata et al,

circulating TNF-α serum levels and suppressed TNF-α release from colonic supernatants post-DSS induction (Figure 6A-C). Although MMP-9 is a downstream target of plasmin, plasma PAP levels were low in DSS-induced colitic MMP-9⁻/⁻ mice (Supplementary Figure 9). These data indicate that plasmin is important in mounting a cytokine response.

**Reduced CXCL5 release during colitis after plasmin and MMP-9 inhibition**

In vivo processing of chemokine (C-X-C motif) ligand 5 (CXCL5) by MMP-2 and MMP-9 promotes neutrophil recruitment in a model of peritonitis.²⁰ CXCL5 gene expression did not change, but YO-2 treatment or MMP-9 genetic deficiency prevented a rise in serum CXCL5 levels in DSS-induced colitic mice (Figure 6D). We investigated whether CXCL5 is released from colonic tissues and might be involved in leukocyte migration. The improved myeloid (U937) cell migration towards supernatants derived from colitic mice was due to a plasmin/MMP-9-dependent production of CXCL5 as shown using neutralizing antibodies (Abs) (Figure 6E). These data demonstrate that plasmin enhances the colon-derived release of CXCL5, thereby enhancing neutrophil influx.

**CD11b⁺ cells drive plasmin-mediated colitis progression**
Munakata et al,

If the recruitment of CD11b$^+$ cells is the main function of plasmin-mediated effects during colitis, blocking CD11b cell recruitment using CD11b neutralizing antibodies (Ab) should improve clinical signs of colitis. But this was not the case (Figure 7A). Similar to previous reports,21 80% of DSS colitis induced mice treated with neutralizing Abs against CD11b succumbed (Figure 7A), although CD11b$^+$Gr-1$^+$ cell influx into colonic tissues (Figure 7B) and local colonic TNF-α production was blocked (Figure 7C). Mononuclear cells isolated from blood of CD11b Ab, but not YO-2 treated mice showed high TNF-α mRNA expression (Supplementary Figure 9). Systemic TNF-α increase correlated with the systemic increase in mononuclear cells, whereas local colonic TNF-α production correlated with the number of infiltrated CD11b$^+$ cells. Survival and WBC counts were restored and histopathological changes and TNF-α serum level elevation (Figure 7D-E) were suppressed after co-injection of YO-2 with CD11b Abs (Figure 7A-D). Our data suggest that plasmin promotes colitis progression by accelerating the systemic inflammatory response (leukocytosis) and leukocyte activation resulting in the release 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 immunological/inflammatory response during colitis progression by accelerating the influx of CD11b$^+$ myeloid cells, and preventing their activation causing
Munakata et al,

the release of critical inflammatory cytokines responsible for tissue destruction.

DISCUSSION

In this study, we provide genetic, functional, and biochemical evidence that the orderly activation of two separate protease systems, the Plg/PA 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 TNF-α addition to myeloid cells increased the expression of uPA in vitro. We found local upregulation of the urokinase plasminogen activator in inflamed, but not control, colonic tissues. The observed low uPA expression in colitic tissues in plasmin inhibitor-treated mice could be due to the impaired infiltration of uPA-producing CD11b+ cells and/or the blockade of local and system TNF-α increase. Our data demonstrating PAP elevation in DSS-treated colitic mice are in accordance with clinical reports from patients with IBD. The presence of fibrinolytic factors within colonic tissues, e.g. uPA expressing myeloid cells suggest that localized proteolysis occurs during colitis, paving the way
Munakata et al,

for inflammatory cells or intestinal microbes to further induce tissue damage. Our data are schematically represented in Figure 7F.

The fibrinolytic system is activated in patients with IBD, this is especially true for those with active disease. Our initial decision to explore the therapeutic potential of the YO-2 inhibitor with various forms of experimental colitis was motivated by previous studies showing that administration of the antifibrinolytic agents epsilon aminocaproic acid and tranexamic acid improved clinical outcome of patients with ulcerative colitis. Here, we show that plasmin inhibition completely restored colitis-induced tissue damage, and the clinical outcome in colitic mice.

What are potential downstream targets of plasmin during the colitis process? We could show that the activation of MMPs like MMP-9 is a critical downstream event during colitis progression. Plasmin has been shown to activate MMP-9 in other disease models, including nerve injury, the disease progression of acute graft versus host disease after bone marrow transplantation, and hematopoietic and ischemic tissue regeneration. It has been well established that MMP-9 is consistently upregulated in both animal models and human inflammatory bowel disease, and is associated with disease severity. Confirming data by Castaneda et al., we showed that MMP-9-/- mice exposed to DSS showed a reduced severity of colitis. Epithelial MMP-9, not infiltrated neutrophil-derived MMP-9, has been shown to induce tissue damage during colitis.
Munakata et al,

Here, we found that plasmin inhibition prevented MMP-9 activation and TNF-α release both systemically in the circulation and locally in the colonic environment. TNF-α release involves a process called shedding involving the protease ADAM-17/TNF-α converting enzyme, but also other MMPs like MMP-1, -7 and -9. Our data indicate that plasmin-dependent TNF-α production during colitis requires endogenous MMP-9, which either directly or by activating other proteases promote TNF-α production. But YO-2 also seems to have MMP-9 independent means of action, because YO-2 treatment improved survival in MMP-9−/− mice. Because plasmin can activate other MMPs like MMP-3, -7, and -12, which are linked to the progression of colitis, we suspect that the control of other MMPs might responsible for the improved survival of MMP-9−/− mice treated with YO-2. But further studies will be necessary to identify those.

Anti-inflammatory treatment medication is a mainstay in IBD treatment. Here, we show that DSS readily induced the expression and raised serum levels of the inflammatory cytokines IL-1β and IL-6. Infliximab, a monoclonal Abs to TNF-α, appeared to be a good therapeutic agent for IBD patients. However, Abs treatment can lead to infusion reactions, loss of response, and serum sickness, aside from its high expense. Therefore, use of small molecules like the plasmin inhibitor YO-2 would not only be cheaper, but might even be safer as there is less concern about immunogenicity in patients.

The influx of CD11b+ macrophages and neutrophils into the inflamed tissue is a critical
Munakata et al,

pathogenic aspect of IBD, and we showed here that both cell types are recruited into colon tissues in a plasmin-dependent manner. CD11b interacts with the intercellular adhesion molecules ICAM-1 and ICAM-2, which have been suggested as therapeutic targets in colitis. We showed that although CD11b Ab treatment slightly improved survival in colitis mice, the antibody could not control the systemic TNF-α release, and resulted in peripheral leukocytosis. These data are consistent with other inflammatory models, like peritonitis, where CD11b Ab treatment prevented myelomonocytic cell recruitment in vivo.

We previously demonstrated that plasmin regulates Gr-1+ neutrophil infiltration during hindlimb ischemia recovery. Here, we found that plasmin inhibition prevented the infiltration of Gr-1+ neutrophils into colonic tissues. Gut enterocyte–derived CXCL5 can attract CXCR2+ neutrophils into the gut tissues. Recently, it was shown that in vivo processing of CXCL5, MMP-2 and MMP-9 promotes early neutrophil recruitment in IL-1β-induced peritonitis. We found increased CXCL5 gene expression in colonic tissues of DSS-induced colitis mice. Circulating CXCL5 protein levels were suppressed both in YO-2 treated mice and MMP-9−/− mice, demonstrating that plasmin inhibition (most likely via MMP inhibition – but further studies are necessary) can control CXCL5 production, thereby preventing influx of MMP-9-carrying neutrophils.

Our data support a mechanism whereby activation of plasminogen during colitis
Munakata et al,

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 approach is prophylactic, but in the future, we are extremely desirous that YO-2 is a new therapeutic target for dysfunction in IBD with an inflammatory component.

Materials and Methods

Animal studies. MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice and Plg$^{+/+}$ and Plg$^{-/-}$ mice were each used after 10 week back crosses onto a C57BL/6 background. C57BL/6 Rag2$^{-/-}$ (recombinase-activating gene 2 deficient) 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. (A) Experimental DSS (ICN Biomedical molecular weight =36000-50000Da) colitis was induced by administering 2% DSS via drinking water on days 0-7. TNBS-induced colitis was induced by colonic injection of 100 μL of 2.5% trinitrobenzene sulfonic acid (TNBS; Sigma, St. Louis, MO) dissolved in 50% ethanol. The control group received only 50% ethanol. (B) Anti-CD40 IBD mouse model:
Munakata et al,

C57BL/6 Rag2^{−/−} mice were administered intravenously with a single injection of 200 μg of anti-CD40 (IgG2a, monoclonal Ab FGK45).\textsuperscript{35, 36}

YO-2 (YO-2, \textit{trans}-4- Aminomethylcyclohexanecarbonyl-Tyr(O-Pic)-octylamide\textsuperscript{37} (kindly provided by Yoshio Okada, The Faculty of Pharmaceutical Science, Kobe Gakuin University) was administered by intraperitoneal (i.p.) injection, once per day at 4mg/kg/day from day 0-28. The tranexamic acid moiety of YO-2 interacts with the active center of plasmin and plasma kallikrein. The MMP inhibitor [N-hydroxy-2-((4-methoxysulfonyl)(3-picolyl)-amino)-3- methylbutanamide (MMI270)] (Novartis Pharma Corporation, Basel, Switzerland)\textsuperscript{38} was orally administered at a dose of 100mg/kg/day from day 0-7. CD11b neutralizing antibody treatment: mice were injected with the 500 μg/mouse of anti CD11b antibody (clone 5C6) or control immunoglobulin G (IgG) on day 0, 2, and 4.

The clinical scoring of a disease activity index for DSS-induced colitis was based on weight loss, stool consistency, and bleeding, as described previously by Cooper et al.\textsuperscript{39}. Stool scores were determined as 0 = normal stools; 2 = loose stools; 4 = diarrhea. Bleeding scores were determined as 0 = no bleeding; 4 = + macroscopic bleeding.

\textit{Histology.} Colons were stained with hematoxylin and eosin (H&E) and Elastica van Gieson staining. Slides were scored blindly by 2 individuals based on a scale that graded the extent of inflammatory infiltrate (0-5), crypt damage (0-4), ulceration (0-3), and the presence or absence of edema (0 or 1). Histological scoring was performed as
Munakata et al., described by Cooper et al.\textsuperscript{39}.

**Bleeding time.** The tail of mice was warmed to 37°C, put in a horizontal position and amputated 1 cm from the tail tip. Blood was blotted onto filter paper every 15 second.

**Immunohistochemistry.** Distal colon sections were stained with the anti-Gr1 (clone RB6-8C5; R&D Systems), anti-MMP-9 antibody (R&D Systems), anti-F4/80 antibody (clone A3-1; AbD Serotec), anti-CD11b antibody (clone M1-70; BD Pharmingen), anti-uPA antibody (Proteintech) and anti-fibrinogen antibody (Biogenesis) followed by the antibody conjugated with Alexa 488 and 594 (Invitrogen). Nuclei were stained using DAPI.

**TUNEL assay.** Apoptosis was detected using tissue stained colon section using the instructions provided by the maker of the TAC2 TdT Kit (Funakoshi, Tokyo, Japan).

**Quantification of Cytokine mRNA Expression in Colon Tissues by RT-PCR.**

Total RNA was extracted using Trizol (Invitrogen), and cDNA was generated according to the manufacturer’s protocols. 10 mg of colon was used. Peripheral blood mononuclear cells were isolated by centrifugation using Lympholyte (Cedarlane Inc.) were taken.

PCR were performed by the following specific forward and reverse primer pairs: for 

- **uPA:** (5’-gtctctctctgcaacagagtc-3’) and (5’-ctgtgtctgagggtaatgct-3’), for **tPA:**
  (5’-gtactgttggacct-3’) and (5’-tgctgttggtaatgctg-3’), for **TNF-α:**
  (5’-gccgattgtctatcata-3’) and (5’-ggtatatggtcata-3’), for **IL-1β:**
  (5’-gcaactgttcctgactc-3’) and (5’-ctggagcctgatcag-3’), for **IL-6:**
  (5’-gtatatgagtgactgatgag-3’) and (5’-ccagaaggagacagaaa-3’) for the **b-actin** control:
  (5’-tgacaggatgcaagagaaa-3’) and (5’-gctggagtgactgag-3’), for **CXCL5:**
  (5’-gcattctgttctgatctggtcagctg-3’) and (5’-cctctctctctctgatgtg-3’)

**Immunooassay.** The levels of cytokines were determined in plasma, serum and colon supernatant. Colon supernatants were prepared as described previously.\textsuperscript{40} Samples were
Munakata et al, measured using commercially available mouse-specific ELISA kits for murine MMP-9, CXCL5, TNF-α (all three R&D Systems Inc., Minneapolis, MN), plasmin-antiplasmin complex (PAP) (CUSABIO BIOTECH, Newark, DE), fibrinogen degradation product (FDP) (Uscn life science Inc., Wuhan), Thrombin-antithrombin complex (TAT) (Abcam, Cambridge, MA, USA) and IL-1β, IL-6 (Biolegend, San Diego, CA).

Gelatin zymography. Plasma samples were treated with 20 μl gelatin-agarose beads at 4°C overnight and processed through SDS-PAGE acrylamide gels containing 1 mg ml⁻¹ gelatin. For details see 41.

Transwell migration assay. Serum starved U937 cells (1×10⁶) were placed in 24-well transwell inserts with 8 mm pores (Corning Life Sciences, Lowell, MA) of 24-well plates. Cells migrated for 2 hours at 37°C towards colon supernatants derived from DSS-induced mice treated with or without YO-2 in the presence of IgG and CXCL5 antibody, or RPMI-1640 medium alone. The number of migrated cells was counted.

**Statistical Analyses.** All data are presented as means ± standard error of the mean (SEM). Student’s t-tests were performed. Survival curves were plotted using Kaplan-Meier estimates with log rank. P < 0.05 was considered significant.
Munakata et al,

References


Munakata et al,


Munakata et al,


Munakata et al,


Munakata et al,

Figure Legends

Figure 1. Plasmin inhibition prevents DSS-induced colitis progression. Colitis was induced by DSS in drinking water, and mice were injected with or without YO-2. (A) Plasma derived from mice treated with or without YO-2 was analyzed for plasmin-antiplasmin complex (PAP) as a measure of active plasmin by enzyme-linked immunosorbent assay. n = 3 / group. (B) Percentage survival, (C) disease active index were determined at indicated time points in the following treatment groups of mice: 2% DSS+PBS, n = 15; 2% DSS+YO-2, n = 15; water + PBS and water+YO-2, n = 5. (D) Colon lengths were measured at indicated time points. n = 9-14 for per group. (E) Representative H&E-stained sections of colon tissue are given. Scale bar: 200 μm. Histological inflammatory scores were determined for each colon section from DSS-induced mice treated with/without YO-2. n = 3 / group. Values represent mean ± SEM. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test and log rank test.

Figure 2. Plasmin inhibition suppresses the influx of myeloid cells into colonic tissue. Colitis was induced by DSS in drinking water, and mice were injected with or without YO-2. (A) Representative immunohistochemical images of Gr-1 (neutrophil marker)/CD11b and F4/80 (macrophage marker)/CD11b-stained distal colonic sections
Munakata et al,

retrieved 7 days after DSS-induction of C57BL/6 mice treated with or without YO-2.

Arrows indicate positively stained cells. n = 3 per group. Scale bar: 200 μm. Right Panels: indicated cell populations were quantified per high power field (HPF). (B) Urokinase plasminogen activator (uPA) gene expression in whole colon retrieved from mice treated with or without YO-2 was semi-quantitated by reverse-transcription polymerase chain reaction (RT-PCR). Levels were normalized to β-actin. n = 3 / group.

(C) Staining of colon sections revealed uPA staining of a CD11b+ cell subpopulation. Scale bar: 200 μm. Data represent mean ± SEM. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test.

**Figure 3. Plg-deficient mice are less susceptible to DSS-induce colitis.** (A) Survival and (B) disease active index were determined over the indicated period in DSS-treated Plg+/+ and Plg−/− mice: n = 15 / group. (C) Plg−/− mice exhibit shorter colon lengths. n = 9 for each group. Representative H&E-stained sections of distal colons from DSS-treated Plg+/+ and Plg−/− mice (D) (right panel). Scale bar: 200 μm. n = 3 / group for all experiments. Right panel: pathological scores obtained using colon tissues. Values represent mean ± SEM. Percentage survival was determined. n = 15. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test and log rank test.
Munakata et al,

**Figure 4. Plasmin-mediated prevention of colitis progression requires MMP-9.** (A) Representative immunohistochemical images of Gr-1 (neutrophil marker)/MMP-9-stained distal colonic sections retrieved 7 days after DSS-induction of C57BL/6 mice treated with or without YO-2. Arrows indicate positively stained cells. n = 3 per group. (B) Blood serum samples and (C) culture supernatants derived from colon tissue homogenates of YO-2- or PBS-treated C57BL/6 mice were analyzed by gelatinolytic zymography (left panel). Right panel: Quantification of the proteolytic activity of actMMP-2 and act MMP-9 were detected in colon culture supernatants by densitometry n = 5 / group. (D) Survival was determined in DSS-induced colitic MMP-9+/− and wild-type mice co-injected with YO-2 and MMI270. Values represent mean ± SEM. Survival was determined in the following treatment groups: MMP-9+/+ +PBS and MMP-9+/+ +YO-2, n = 15; MMP-9−/− +PBS and MMP-9−/− +YO-2, n = 15; MMP-9+/+ +MMP270 and MMP-9−/− +MMP270, n = 20. (E) Disease activity index and representative H&E-stained sections (F) of distal colons from DSS-treated MMP-9+/+ and MMP-9−/− colitic mice (right panel) are given. Scale bar: 200 μm. n = 3 / group for all experiments. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test and log rank test.
Figure 5. Plasmin inhibition ameliorates TNBS-induced and CD40-induced colitis.

Colitic mice induced by TNBS administration (data given on the left side) or by CD40 antibody injection into Rag-2-/- mice (data are given on the right side) were treated with PBS and YO-2. (A) Levels of PAP in plasma (A) and (B) TNF-α in serum and in colon supernatants of indicated experimental groups by ELISA. (C) Body weight loss setting starting body weight as 100% in control- and TNBS-treated mice. n = 10 / group. (D) Representative images of distal colon sections from TNBS-induced colitic mice stained with H&E, and immunostained using Gr-1, MMP-9, and F4/80 Abs. Scale bar: 200 μm. (E) Representative images of distal colon sections from CD40Ab-induced colitic mice stained with H&E, and immunostained using Gr-1, CD11b, and F4/80 Abs. 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 mean ± SEM. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test.

Figure 6. Pharmacological plasmin inhibition alleviates systemic and colonic cytokine production in DSS-induced colitic mice. (A) Gene expression of IL-1β, IL-6 and TNF-α in distal colon homogenates of C57BL/6 mice treated with PBS control or YO-2 as determined by PCR (normalized to the expression of β-actin). n = 4 or 5 /
group. (B) The inflammatory cytokines IL-1β and IL-6 were assayed in serum samples of mice with DSS-induced colitis treated with or without YO-2 as determined by ELISA. 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<sup>+/+</sup> or Plg<sup>−/−</sup>, and MMP-9<sup>+/+</sup> and MMP-9<sup>−/−</sup> mice 7 days after the initiation of DSS treatment by ELISA. n = 3-6 for each group. (D) CXCL5 gene 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 MMP-9<sup>−/−</sup> (right panel) or respective littermate controls as determined by ELISA. n = 3-6 / group. (E) Transmigration index of U937 cells migrating towards medium alone, colon supernatants extracted from normal mice (d0), or supernatants from DSS-treated mice coinjected with PBS or YO-2 in the presence or absence of CXCR5 antibody. n = 3-5 / group. Data represent mean ± SEM. *P < .05, **P < .01, determined by 2-tailed Student’s t-test.

**Figure 7. Improved disease control and inflammatory cytokine response in YO-2-treated compared to CD11b antibody-treated mice during experimental colitis.** DSS-induced C57BL/6 mice were treated with YO-2 and coinjected with anti-CD11b or control monoclonal antibodies. (A) Survival rate was determined in the
Munakata et al,

following treatment groups: 2%DSS+PBS, n = 10; 2%DSS+CD11b Ab, n = 15; 2%DSS+CD11b Ab+YO-2, n = 10. (B) Representative H&E-stained sections of distal colons from treated mice. (C) TNF-α protein in supernatants from colon cultures at day 7. (D) The total number of white blood cells (WBC) was counted at indicated time points. n = 6 / group. (E) TNF-α protein was determined in plasma samples taken at day 7. Data represent mean ± SEM. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test and log rank test. (F) A model of various target molecules of plasmin involved in experimentally induced colitis: The secreted latent plasminogen 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-MMP-9 to MMP-9. This generated proteolytic environment not only damages colonic tissues, but also releases proinflammatory cytokines (like TNF-α) and chemokines (like CXCL5) known to promote the influx of CD11b+ inflammatory cells into colonic tissues. These CD11b+ inflammatory cells support the colonic tissue damage in part by again providing necessary proteases to fuel this vicious cycle.
Supplementary Figure 1-10. Mice were treated with DSS in drinking water and treated with or without YO-2. At indicated time points either blood or colon tissues were obtained. Data represent mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, determined by 2-tailed Student’s t-test.

Supplementary Figure 1. The coagulation marker thrombin-antithrombin complex (TAT) is elevated in plasma of control, but not YO-2 treated mice as determined by ELISA at day 7. $n = 3 / \text{group}$.

Supplementary Figure 2. Bleeding time was impaired in both YO-2 and control-treated DSS mice at day 7. $n = 6 / \text{group}$.

Supplementary Figure 3. Fibrin/Fibrinogen immunostaining of colon tissues. No fibrin deposit was detected in normal colon tissues (day 0), in colitic tissues after 7 of DSS ingestion with or without YO-2 and in colitic tissues of mice that had been treated for 28 days with YO-2. $n = 3 / \text{group}$.

Supplementary Figure 4. Fibrin degradation product (FDP) levels in blood samples of mice were analyzed $n = 3-6 / \text{group}$.
Munakata et al,

**Supplementary Figure 5.** TUNEL staining of colon tissues revealed an increased number of TUNEL⁺ cells in the control treated group (left panel). Arrows indicate TUNEL positively stained cells. \( n = 3 \) per group. Right Panel: Quantification of the number of TUNEL⁺ cells per high power field (HPF). Scale bar: 20μm.

**Supplementary Figure 6.** Elastica van Gieson staining (blue staining; used to differentiate between collagen and smooth muscle; Collagen shows Bright red Cytoplasm, muscle, fibrin and red blood cells stain Yellow) of colon tissues obtained from mice 7 days after the start of YO-2 injections.

**Supplementary Figure 7.** MMP-9 serum levels assayed in YO-2- or PBS-treated C57BL/6 mice by ELISA. \( n = 3 \) for each group.

**Supplementary Figure 8.** PAP plasma levels in DSS-treated \( MMP-9^{+/+} \) and \( MMP-9^{-/-} \) mice by ELISA. \( n = 3 \) for each group.

**Supplementary Figure 9.** Gene expression of TNF-α in circulating mononuclear cells of indicated groups at day 7. \( n = 3 \) for each group.
Munakata et al,


Munakata et al.


Munakata et al,


Munakata et al,


