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**Zoobiquity experiments show the importance of the local MMP9-plasminogen axis
in inflammatory bowel diseases in both dogs and patients**

Takeshi Yamasaki^{1,2*}, Noriyuki Nagata^{3*}, Toru Atsumi^{1*}, Rie Hasebe^{2,4*}, Yuki
Tanaka^{1,5*}, Izuru Ohki^{1,5}, Shimpei Kubota¹, Yuta Shinohara¹, Yong Bin Teoh³, Nozomu
Yokoyama⁶, Noboru Sasaki³, Kensuke Nakamura³, Hiroshi Ohta⁷, Takehiko Katsurada⁸,
Yoshihiro Matsuno⁹, Shintaro Hojyo¹, Shigeru Hashimoto¹, Mitsuyoshi Takiguchi^{3*},
and Masaaki Murakami^{1,2,5,10}

¹Division of Molecular Psychoimmunology, Institute for Genetic Medicine, Graduate
School of Medicine, Hokkaido University, Sapporo, 060-0815, Japan

²Division of Molecular Neuroimmunology, Department of Homeostatic Regulation,
National Institute for Physiological Sciences, National Institute of Natural Sciences,
Okazaki, 444-8585, Japan

³Laboratory of Veterinary Internal Medicine, Department of Clinical Sciences, Graduate
School of Veterinary Medicine, Hokkaido University, Sapporo, 060-0818, Japan

⁴Center for infectious Cancers, Institute for Genetic Medicine, Graduate School of
Medicine, Hokkaido University, Sapporo, 060-0815, Japan

⁵Group of Quantum Immunology, Institute for Quantum Life Science, National Institute
for Quantum and Radiological Science and Technology (QST), Inage, 263-8555, Japan

⁶Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido
University, Sapporo, 060-0818, Japan

⁷Laboratory of Veterinary Internal Medicine, Department of Small Animal Clinical Sciences, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, 069-8501, Hokkaido, Japan

⁸Department of Gastroenterology and Hepatology, Graduate School of Medicine, Hokkaido University, Sapporo, 060-8638, Japan

⁹Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, 060-8648, Japan.

¹⁰Institute for Vaccine Research and Development (HU-IVReD), Hokkaido University, Sapporo, 001-0020, Japan

***equal contributed**

Correspondence:

Masaaki Murakami; Kita-15, Nishi-7, Kita-Ku, Sapporo, Hokkaido 060-0815, Japan, +81-11-706-5121, murakami@igm.hokudai.ac.jp

Mitsuyoshi Takiguchi; mtaki@vetmed.hokudai.ac.jp

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ABSTRACT

Using a zoobiquity concept, we directly connect animal phenotypes to a human disease mechanism: the reduction of local plasminogen levels caused by matrix metalloproteinase-9 (MMP9) activity is associated with the development of inflammation in the intestines of dogs and patients with inflammatory bowel disease. We first investigated inflammatory colorectal polyps (ICRPs), which are a canine gastrointestinal disease characterized by the presence of idiopathic chronic inflammation, in Miniature Dachshund (MD), and found 31 missense disease-associated SNPs by whole-exome sequencing. We sequenced them in 10 other dog breeds and found five, *PLG*, *TCOF1*, *TG*, *COL9A2*, and *COL4A4*, only in MD. We then investigated two rare and breed-specific missense SNPs (T/T SNPs), *PLG*: c.477G>T and c.478A>T, and found that ICRPs with the T/T SNP risk-alleles showed less intact plasminogen and plasmin activity in the lesions compared to ICRPs without the risk-alleles but no differences in serum. Moreover, we show that MMP9, which is a NF-κB target, caused the plasminogen reduction and that intestinal epithelial cells expressing plasminogen molecules were colocalized with epithelial cells expressing MMP9 in normal colons with the risk-alleles. Importantly, MMP9 expression in patients with ulcerous colitis or Crohn's disease also colocalized with epithelial cells showing enhanced NF-κB activation and less plasminogen expression. Overall, our zoobiquity experiments showed that MMP9 induces the plasminogen reduction in intestine, contributing to the development of local inflammation and suggesting the local MMP9-plasminogen axis is a therapeutic target in both dogs and patients. Therefore, zoobiquity-type experiments could bring new perspectives for biomarkers and therapeutic targets.

INTRODUCTION

Zoobiquity originated from the One Health Initiative, which was designed to increase collaboration between various disciplines of medicine including veterinary medicine. From this effort, many cross-species risk factors were discovered, such as the BRCA1 genetic mutation, which increases the risk of breast cancer in jaguars and humans (1,2). Other examples include obsessive-compulsive disorder in dogs, high rates of chlamydia in koalas, and horses plagued by self-harming behavior (3). These observations suggest new therapeutic targets for human diseases will come from the study of animal diseases. However, no report directly connects animal phenotypes to a human disease mechanism in non-zoonotic diseases.

Inflammatory colorectal polyps (ICRPs) refer to a canine gastrointestinal disease that affects the descending colon and rectal mucosa. ICRPs cause idiopathic chronic inflammation and show an infiltration of immune cells from the blood along with tissue granulation (4). The most notable characteristic of ICRPs is their apparent breed-specificity, with Miniature Dachshund (MD) accounting for more than 80% of cases in Japan, strongly suggesting the involvement of genetic and environmental factors including special food intake and gut microbiota alterations (4-6). Based on these unique characteristics, ICRPs are considered a breed-specific inflammatory bowel disease (IBD) (7). Despite the high suspicion of genetic susceptibility, the disease-associated SNPs of ICRPs have not been well studied. Previous research has reported an association between the nucleotide-binding oligomerization domain 2 (NOD2) gene, a pattern recognition receptor, which is also associated with Crohn's disease in humans (5). However, multiple genes, including common variants with low effect and rare variants with high effect, are associated with complex diseases such as IBD (8,9).

Furthermore, whole-exome sequencing and whole-genome sequencing have revealed rare and high impact variants in complex diseases including IBD in humans.

The *PLG* gene encodes plasminogen, which is a zymogen form of serine protease plasmin. Plasminogen is composed of seven domains including a Pan-apple domain, five kringle domains and a serine protease domain (10). Although the main function of the plasminogen-plasmin axis is believed to be fibrinolysis, the axis is actually involved in many processes including cell migration, embryogenesis, angiogenesis, and wound healing (11-13). Interestingly, plasminogen deficiency in humans is not associated with an increased risk of thrombosis, but it is associated with wood-like pseudomembranous or ligneous conjunctivitis on the mucosa, most likely due to impaired fibrin clearance and wound healing (14-16), suggesting that inflammation processes are disrupted. At least 25 missense SNPs have been reported for the few dozen plasminogen deficiency cases reported (15,16). An analysis of SNPs in the *PLG* gene demonstrated that most of these variants are related to the impaired secretion or instability of plasminogen molecules, resulting in plasminogen deficiency (16). Some variants are located on kringle domains, suggesting the instability of plasminogen molecules is due to improper kringle folding (16). A previous study on the genetic risk factors in human patients with multiple sclerosis, a well-known chronic inflammatory disease of the central nerve system, showed that a missense SNP in *PLG* gene (p.G420D) is associated with the onset of the disease (17), suggesting that plasminogen molecules are involved in the inflammation development. Consistently, plasminogen activators and their inhibitors were present in intestinal lesion of IBD patients (18) and *PLG*-deficient mice were partly protected against colitis (19). However, no report describe how *PLG* is activated in local regions.

We previously found that activated immune cell-derived cytokines, such as IL-6, TNF- α and IL-17A, enhanced the expression of NF- κ B targets, including inflammatory cytokines and chemokines, particularly in nonimmune cells, leading to the development of tissue-specific inflammatory diseases (20,21). We named this machinery the IL-6 amplifier. Mechanistically, the IL-6 amplifier is stimulated by the simultaneous activation of NF- κ B and STAT3 to hyper-activate NF- κ B. IL-6 is an exclusive STAT3 stimulator, but several NF- κ B stimulators, including TNF- α , IL-17A, and growth factors, have been observed during local inflammation development together with activation of the IL-6 amplifier (20,22). Since its original discovery, evidence of the IL-6 amplifier has been found in many mouse disease models and patient samples of several diseases including arthritis, multiple sclerosis, psoriasis, uveoretinitis, and chronic rejection (21,23-38). A later genome-wide screening to identify the genes that regulate the IL-6 amplifier found about 1,200 positive regulators and 500 target genes (20,35). We also found that MMP9 molecules are target candidates of the IL-6 amplifier (35).

To perform zoobiquity experiments, we first identified disease-associated SNPs for ICRPs in MDs using whole-exome sequencing, focusing especially on rare and breed-specific variants. Our data suggested that missense SNPs in *PLG* gene are associated with ICRPs, because PLG molecules in the lesions were reduced, most likely in a manner dependent on MMP9. MMP9 expression in patients with ulcerous colitis or Crohn's disease was similarly colocalized with epithelial cells with an enhanced-activation of NF- κ B and reduced plasminogen expression. Thus, the local MMP9-plasminogen axis may contribute to the development of inflammation in the intestine of

141 dogs and humans. These findings suggest zoobiquity-type experiments will bring new
142 perspectives for biomarkers and therapeutic targets.

MATERIALS AND METHODS

Animals

The diagnosis of ICRPs was based on previously established histological findings (4). Blood and colon tissue samples were collected from MDs with or without ICRPs, dogs of various breeds, and laboratory beagles (Supplementary Tables 1 and 2). A signed consent form was obtained from all owners of the clinical cases. All procedures were approved by the Hokkaido University Institutional Animal Care and Use Committee.

DNA Extraction

Genomic DNA (gDNA) was derived from the dog samples using a DNA Blood & Tissue Kit (QIAGEN, Hilden, Germany).

Whole-Exome Sequencing

For exome capturing, SureSelect Canine All Exon Coverage (Agilent Technologies, Wilmington, DE, USA) was used. Sequencing was performed using an Illumina HiSeq2500. Data quality was checked using the FASTQC program. Low-quality bases (quality score of < 20) were trimmed from the 3'-end, and low-quality reads were filtered. Then the sequence reads were mapped to the dog genome assembly canFam3 (Broad CanFam3.1, September 2011) downloaded from the UCSC Genome Browser using BWA (0.7.6a). The mapped reads were submitted for post-processing analysis using Picard tools (1.129) and the Genome Analysis Toolkit (GATK 3.3). HaplotypeCaller utility in GATK was used for variant calling. Functional annotation of

the variants was carried out using ANNOVAR, with CanFam3 as the dog gene annotations.

Sanger Sequencing

Direct Sanger sequencing was performed on 31 variants selected from the results of the whole-exome sequencing analysis. Primer pairs were designed for each variant using Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>; Supplementary Table 3). After the PCR reactions, the amplicons were purified using ExoSAP-IT Express PCR Cleanup Reagents (Thermo Fisher Scientific, Waltham, MA, USA) and then submitted for Sanger sequencing with BigDye Terminator v3.1 (Thermo Fisher Scientific) and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA).

Over-expression of Recombinant Canine Plasminogen and Serine Proteases

A canine plasminogen expression vector was prepared, and point mutations of *PLG* c.477G>T (p.E159D) and *PLG* c.478A>T (p.N160Y) were generated using PCR-based mutagenesis. His-tagged canine matrix metalloproteinase-2 (MMP2), MMP3, MMP9, MMP12, urokinase plasminogen activator (PLAU), and cathepsin D (CTSD) expression vectors were also generated. Plasminogen and each His-tagged vector were transiently co-transfected into HEK293T cells. Twenty-four hours after the transfection, the cells were lysed in lysis buffer containing 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP40, and 3 mM EDTA with protease inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA)

Immunoblot Analysis

Serum was diluted 81 times, and 10 μ L of diluted serum was separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). Colon tissue samples were lysed in lysis buffer, and 20 μ g of total protein was run. Blocking was performed using 5% nonfat milk for 1 hour at room temperature. The primary antibodies used in this study were rabbit anti-plasminogen antibody (1:4,000; proteintech, Tokyo, Japan) and rabbit anti-canine plasminogen specific for E159D and N160Y (against amino acids 152-163 of canine plasminogen, CRNPDNDDYGPW, 1:4,000; Cosmo Bio Co., Tokyo, Japan). The protein signals were detected using ECL (Chemi-Lumi One L, nacalai tasque). The intensity of the bands corresponding to intact plasminogen molecules was analyzed using ImageJ (National Institutes of Health, Bethesda, MD). The intact plasminogen levels of the samples compared to the average level of samples with non-risk-alleles are shown in the figures.

Serine Protease Zymography

Plasmin activity of the serum and tissue samples was analyzed using a Serine Protease Zymography Kit (Life Laboratory, Yamagata, Japan) according to the manufacturer's instructions. For serine protease zymography, serum diluted 20 times or 10 μ g of total protein of lysed colon tissue samples with 8 ng of human plasmin (Biovision, Milipitas, CA, USA) as a standard were loaded on the gels, and electrophoresis was conducted. After the gels were incubated in washing buffer with shaking at room temperature for 1 hour, they were reacted with reaction buffer at 37°C for 24 hours. Then the gels were stained with staining solution at room temperature for 30 minutes, followed by destaining. The plasmin activities were detected as clear bands.

The gels were scanned, and the intensity was analyzed using ImageJ by comparing with standard human plasmin. The intensities of the bands are shown as arbitrary units (AU).

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections using rabbit anti-plasminogen antibody (1:2,000; proteintech), rabbit anti-canine plasminogen specific for E159D and N160Y (against amino acids 152-163 of canine plasminogen, CRNPDNDDYGPW, 1:2,000), rabbit anti-MMP9 antibody (1:6,400; Abcam), rabbit anti-phospho-STAT3 antibody (Tyr705; 1:800; Cell Signaling Technology), and rabbit anti-phospho-p65 antibody (Ser536; 1:800; Sigma-Aldrich). For quantification of the plasminogen signals, the signal area of 10 signals/section was analyzed using ImageJ software.

In addition to the canine tissue samples, immunohistochemistry was performed on paraffin sections of human patients with inflammatory diseases in the intestine such as ulcerous colitis and Crohn's disease. For the human samples, positive areas in the colon epithelium were quantified using ImageJ. The percentage of positive areas in the lumen structures was calculated from 12 lumen structures for each patient ($\times 10$ objectives).

The protocol was approved by the ethics committee of the Hokkaido University Graduate School of Medicine.

Real-time PCR

The CFX96 Touch Deep Well Real-Time PCR Detection System (BIO-RAD) and SYBR Green PCR master mix (Kapa Biosystems) were used to quantify the expression levels of target mRNA and internal control mRNA glycerol-3-phosphatase

dehydrogenase (GAPDH) for H4 human cells. The cells were plated in 96-well plates (7×10^3 cells/well) and stimulated with 100 ng/mL of human IL-6 (Toures) plus 100 ng/mL of soluble IL-6R (R&D Systems) and/or 20 ng/mL of TNF- α (PeproTech) for 3 hours after 2 hours of serum starvation. Total RNA was prepared from the cells using a SuperPrep Cell Lysis for qPCR (TOYOBO). The PCR primer pairs used for the real-time PCR were human IL-6 GGTACATCCTCGACGGCATCT and GTGCCTCTTTGCTGCTTTCAC, human MMP3 CAAAACATATTTCTTTGTAGAGGACAA and TTCAGCTATTTGCTTGGGAA, and human MMP9 GAACCAATCTCACCGACAGG and GCCACCCGAGTGTAACCATA.

The conditions for real-time PCR were 40 cycles at 95°C for 3 s followed by 40 cycles at 60°C for 20 s. The relative mRNA expression levels were normalized to the levels of GAPDH mRNA.

Statistical Analysis

Two-tailed Student t test and Fisher's exact test were used to analyze differences between two groups. One-way ANOVA was used for multiple comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

Identification of five MD-specific SNPs in the coding regions of genomic DNA

To identify the gene(s) responsible for the ICRP pathology in MDs, we performed whole-exome sequencing on two ICRP-affected MDs (ICRP-MDs) and two laboratory beagles. We found 31 missense SNPs in 25 genes, including *NOX3*, *PLG*, *TCOF1*, *ITGAD*, *CDH2*, *PTGER2*, *TSHR*, *GGTA1*, *ARHGAP8*, *PDGFB*, *UBE2N*, *DLA-64*, *TG*, *COL9A2*, *CYP4A11*, *RNASEH1*, *cOR52N9*, *GIF*, *CD40*, *IL29L*, *COL4A4*, *ZDHHC8*, *KLRA1*, *RPL4* and *FGF5*, were present in ICRP-MDs but not in beagles (Supplementary Table 4). We then sequenced the 31 SNPs in 10 other dog breeds: Cavalier King Charles Spaniel, Chihuahua, Jack Russell Terrier, Labrador Retriever, Miniature Schnauzer, Papillon, Shiba, Shih Tzu, Toy Poodle, and Welsh Corgi. Five SNPs, *PLG*, *TCOF1*, *TG*, *COL9A2*, and *COL4A4*, were not found in these ten breeds.

Two *PLG* SNPs are associated with the ICRP pathology in MDs

Because plasminogen is reported to be associated with inflammation, we focused on *PLG* SNP: c.478A>T (p.N160Y), which was present in two ICRP-affected MDs (Supplementary Table 4). The frequency of c.478A>T variant, including homozygote and heterozygote, was significantly higher in ICRP-MDs (n = 40) than control-MDs, which did not have the intestine pathology (n = 43) ($P = 0.002$). All homozygotes were present in ICRP-MDs (Figure 1). When direct sequencing was performed for the SNPs in the *PLG* gene, another SNP, *PLG* c.477G>T (p.E159D), was detected in all cases with *PLG* c.478A>T (p.N160Y), which indicates that these two SNPs have a complete linkage (Figure 2). The alignments of the amino acids among several mammals showed

that both amino acids were not conserved (Figure 2). However, these SNPs were not found among human genome sequences (data not shown).

Plasminogen molecules and plasmin activities were decreased in colon lesions isolated from ICRP-MDs having the risk-alleles

Because most missense variants in human *PLG* gene cause plasminogen deficiency systemically, we investigated serum plasminogen levels in ICRP-MDs with or without *PLG* SNPs. The plasminogen levels in sera isolated from ICRP-MDs were the same with or without the risk-alleles (Figure 3A). So too were serum plasmin activities (Figure 3B and Figure 3C). However, we found that intact plasminogen (82 kDa) molecules were fewer in MDs having the risk-alleles (Figure 4A), suggesting that SNP-mediated amino acid substitutions in the plasminogen molecules induce their degradation in the lesions. Consistently, plasmin activities in the lesions were reduced in ICRP-MDs with the risk-alleles (Figure 4B and Figure 4C). Immunohistochemistry experiments showed that plasminogen molecules were detected in colorectal epithelial cells in the ICRP-lesions (Figure 5A), but the signal area was lower in dogs expressing the SNPs ($P = 0.008$) (Figure 5B). It is known that plasminogen molecules bind to various plasminogen receptors (39,40). We hypothesized that plasminogen molecules with risk alleles bind to different receptors, leading to different localization, as seen in Figure 5A. To detect plasminogen molecules with SNP-mediated amino acid substitutions directly, we established a rabbit polyclonal antibody against amino acids 152–163. Intact and low molecular-weight bands of plasminogen molecules were detected only in lesions with the risk-alleles (Figure 6A). Furthermore, low molecular-weight plasminogen molecules were detected in lamina propria cells only in the lesions

with the risk SNPs (Figure 6B and Figure 6C). These results show that the amino acid substitutions induced by the risk SNPs were correlated with the lower number of plasminogen molecules in the lesions of ICRPs.

MMP9 is induced by the IL-6 amplifier in epithelial cells of the colon to cleave plasminogen molecules with the risk-alleles

Because proteases cleave plasminogen molecules (41), we hypothesized that the risk-alleles increase the probability that plasminogen molecules are targeted by proteases. We therefore expressed several proteases, including MMP12, PLAU, CTSD, MMP2, MMP3, and MMP9, in 293T cells in the presence of plasminogen molecules with or without the risk-alleles and investigated plasminogen degradation. We found that MMP9 cleaved intact plasminogen molecules with the risk-alleles more than those without in vitro (Figure 7A), suggesting that MMP9 is at least one of several enzymes that digest plasminogen molecules. We also found that not only IL-6 but also MMP9 were significantly enhanced by activation of the IL-6 amplifier in vitro (Figure 7B). Interestingly, intestinal epithelial cells with relatively normal structure and expressing MMP9 were colocalized with or localized near epithelial cells expressing nuclear phospho-STAT3, phosphor-NF- κ B molecules and plasminogen molecules in colons with the risk-alleles but not in colons without the alleles (Figure 7C and Figure 7D). On the other hand, it was difficult to investigate the co-localization of MMP9, phospho-STAT3, phosphor-NF- κ B, and plasminogen molecules in polyp lesions with the risk-alleles due to severe inflammation (Supplementary Figure 1). Although we hypothesize that intestinal cells have diverse sensitivity to IL-6 and TNF- α , those with the risk alleles may reduce plasminogen levels and activate the IL-6 amplifier in response to IL-

6 and TNF- α , acting as a starting point for local inflammation. Consistently, we showed that MMP9, pSTAT3, and pp65 are colocalized in ICRP-MDs with the risk alleles as shown in Figure 7. These findings suggest that MMP9 expressed by the IL-6 amplifier cleaved plasminogen molecules in intestines with the risk-alleles.

Higher amounts of MMP9, phospho-STAT3, and phosphor-NF- κ B, and lower amounts of plasminogen molecules exist in intestine lesions of patients with ulcerous colitis or Crohn's disease

Although we do not find the similar plasminogen SNPs in patients with intestinal bowel diseases, we here investigated whether the MMP9-plasminogen-IL-6 amplifier axis identified in the intestine of MDs is also present in some IBD patients. Moreover this axis in MDs was triggered by the plasminogen risk alleles, however, we hypothesize that genetic and/or environmental factors trigger the activation of the axis in human cases. We then performed immunohistochemistry on samples from several patients with ulcerous colitis or Crohn's disease (Table 1). Increased amounts of MMP9, phospho-STAT3, and phosphor-NF- κ B and decreased amounts of plasminogen molecules were found in the intestine lesions of these patients (Figure 8). These observations suggest that the local MMP9-plasminogen axis plays a role in the development of ulcerous colitis and Crohn's disease and that the findings in MDs represent a general concept for the disease pathogenesis.

DISCUSSION

To perform zoobiquity experiments, we first identified that two rare and breed-specific missense SNPs, *PLG*: c.477G>T and c.478A>T, whose frequencies are significantly higher in MDs suffering from ICRPs. We also showed that the SNPs do not cause true hypoplasminogenemia or dysplasminogenemia but increase the degradation of plasminogen molecules in the ICRP lesions, most likely by MMP9 cleavage. This conclusion is based on four findings. First, plasminogen levels and plasmin activity in sera isolated from ICRP-MDs with or without the SNPs were the same. Second, lower intact plasminogen expression and plasmin activity were found in the lesions of ICRP-MDs with the SNPs. Third, cleaved plasminogen molecules were present only in lesions with the SNPs. Lastly, MMP9 cleaved plasminogen molecules with amino acid substitutions induced by the risk SNPs. Because hypoplasminogenemia causes inflammatory diseases, including ligneous conjunctivitis, in humans, our findings suggest that reduced plasminogen levels in the ICRP lesions may explain the high prevalence of ICRPs in MDs. We next investigated whether a similar pathogenesis occurs in human diseases and showed that increased amounts of MMP9, phospho-STAT3, and phospho-NF- κ B and decreased amounts of plasminogen molecules were found in intestine lesions of patients with ulcerous colitis or Crohn's disease. Therefore, the local MMP9-plasminogen axis may play a role in the development of IBD even in humans.

The most important point we learned from the plasminogen deficiency in patients and animal models is that the reduction of plasminogen induces inflammatory diseases in several organs depending on the genetic and/or environmental conditions (14-16). Therefore, a risk allele-mediated reduction of plasminogen in the intestine of

MDs may induce inflammation when accompanied by increased MMP9 and other inflammatory signals in the intestine. However, more study of the IL-6 amplifier-MMP9 pathway is required to know the full details particular of its triggering mechanism.

Although genetic studies in human patients with IBD have identified more than 200 disease-associated loci so far, only few genes have been shown to be causative (42,43). The diversity of the pathology, including ulcerous colitis and Crohn's disease, and the multifactor involvement, such as genetic factors and environmental factors, make it difficult to discover causative loci and to establish the related pathogenesis. The situation with canine IBD might be similar to human cases, although several breeds, such as Weimaraner, Rottweiler, German Shepherd, Border Collie, and Boxer, have been shown to be more susceptible to IBD, with odds ratios of 1.7 to 3.7 (44). ICRPs are quite breed-specific, with an odds ratio of 24.6 (4), which makes this disease a good candidate for studying genetic susceptibility. We found that the SNP-mediated plasminogen reduction is involved in the development of ICRPs via IL-6 amplifier-induced MMP9 expression in the intestines of MDs. Moreover, some samples from patients suffering from ulcerous colitis or Crohn's disease showed similar phenotypes in the intestines. Therefore, ICRPs in MDs may be used for zoobiquity research to identify therapeutic targets for IBD in humans.

The number of patients is still small in our experiments, although subgroups of human IBD may be induced by a molecular mechanism similar to that of ICRPs in MDs via local reduction of plasminogen, because systemic plasminogen-deficiency can induce tissue specific inflammatory diseases in patients (14-16). More study of patient experiments is required to know how the local MMP9-plasminogen axis is involved in the development of IBD in human cases.

How is the IL-6 amplifier activated in the intestine of MDs with risk-alleles? We hypothesized that genetic factors and environmental factors, such as nutrition, gut microbiota, and housing conditions, may activate the IL-6 amplifier in MDs with the risk-alleles. It is reported that the fecal microbiota of MDs with ICRP are significantly altered compared with that of healthy MDs and MDs with ICRP after immunosuppressive therapy (6). This study further showed that the proportions of the phyla Actinobacteria and Fusobacteria were increased in MDs with ICRP, suggesting that the imbalance in gut microbiota is associated with ICRP pathology. Consistently, Fusobacterium species are known to be associated with IBD and the development of colorectal cancer in humans (45,46). Considering these reports, we hypothesize that the decrease in plasminogen molecules in the lesions of ICRP-affected MDs does not affect the gut microbiota directly, but the changes in the composition of gut microbiota could be a risk factor and trigger for the activation of the MMP9-plasminogen axis in MDs with risk-alleles.

We previously reported some of the genes identified in the present study are possible positive regulators of the IL-6 amplifier (35). Therefore, it is possible that genetic and/or environmental factors activate the IL-6 amplifier in colon tissues having the risk-alleles to induce the MMP9-cleavage of plasminogen molecules with the amino acid substitutions. Similarly, the reduction of intact plasminogen in the intestine that develops ICRPs does so most likely with an enhanced activation of the IL-6 amplifier. Regarding the development of ulcerous colitis and Crohn's disease, we consider both genetic and/or environmental factors may activate the IL-6 amplifier in colon tissues like ICRPs.

We hypothesize that NF-kB activation in the colon, which is triggered by genetic and environmental conditions, increased MMP9, a NF-kB target, to reduce the number of plasminogen molecules in dogs with the risk alleles, although the risk SNP does not increase the catalytic activity per a MMP9 molecule. Therefore, it is possible that a certain amount of NF-kB stimulators, including TNF- α and a STAT3 activator, IL-6, both of which are downstream of the NF-kB pathway, are induced from the early phase in a positive feedback response. However, for the development of the inflammatory pathology, NF-kB activation must cross a threshold, and induction of the IL-6 amplifier by the dysregulation of plasminogen activity is one such molecular mechanism for that crossing. Consistently, we showed the positive correlation between the levels of MMP9, pp65, pSTAT3, and the negative correlation between the levels of MMP9 and plasminogen in the intestines of ICRP-MDs and human IBD patients as shown in Supplementary Figure 2 and Supplementary Figure 3.

Plasminogen deficiency in humans and mice is associated with inflammation development in the mucosa, most likely due to impaired fibrin clearance and wound healing (14-16). These findings suggest that inflammation processes are disrupted with less plasminogen function. Therefore, it is possible that fewer normal plasminogen molecules induce inflammation development. Consistently, almost 60% of ICRP-MDs were MDs with at least one risk allele, as shown in Figure 1. On the other hand, we hypothesize that artificial overexpression of the fragmented plasminogen in normal cells does not trigger the inflammation development because there is enough amount of normal plasminogen molecules. Moreover, because fragmented plasminogen molecules should have reduced function, we hypothesize that their overexpression or

supplementation in plasminogen-deficient conditions causes intestinal inflammation and may lead to polyps.

We here showed that MMP9 directly digested plasminogen, leading to the dysregulation of plasminogen-mediated regulatory activity and activation of the IL-6 amplifier in intestine in cases of the risk alleles. Additionally, it is known that tissue-type plasminogen activator (tPA) induces the expression of MMP9 via the NF-kB pathway in inflammatory conditions (47,48). Moreover, the expression of tPA increases in inflammation lesions (49). Therefore, it is possible that the MMP9-plasminogen axis, which we identified here, is enhanced by the tPA-NF-kB axis in intestines, resulting in IBD.

In conclusion, we identified rare and breed-specific SNPs that are associated with ICRP pathology and may be involved in the pathogenesis of disease in MDs. Amino acids substitutions induced by the SNPs promote cleavage by MMP9 to reduce the level of plasminogen in the lesions, resulting in the development of inflammation. A similar scenario is possible in the intestine of some patients with ulcerous colitis or Crohn's disease. Therefore, zoobiquity-type experiments could bring new perspectives for biomarkers and therapeutic targets.

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FOOTNOTES

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681

FIGURE LEGENDS

Figure 1. A *PLG* SNP is associated with the ICRP pathology in MDs

The frequency of *PLG* c.478A>T (p.N160Y) in ICRP-MDs (n = 40), control-MDs (n = 43), and other breeds (non-MDs; n = 40). Its frequency in ICRP-MDs was high compared to that in control-MDs ($P = 0.002$).

Figure 2. Polymorphism of *PLG* genes

(A) A sequence chromatogram of *PLG* c.478A>T (p.N160Y) in wild-type, heterozygote and homozygote samples.

(B) A schema of the canine *PLG* gene and plasminogen protein.

(C) An alignment of amino acid sequences in the kringle 1 domain of plasminogen including variant positions. UniProt (<https://www.uniprot.org/>) was used to align amino acids among several mammals. * completely conserved. : similar properties.

Figure 3. Plasminogen molecules and plasmin activities in sera isolated from ICRP-MDs with risk-alleles were normal

(A) Western blots of sera from 6 ICRP-MDs with non-risk-alleles (A/A) and risk-alleles (T/T) of *PLG* c.478A>T. An antibody for plasminogen (the epitope of amino acids 461–810) was used. An image of intact plasminogen molecules (82 kDa) and the relative levels of intact plasminogen molecules are shown. Data are representative of two independent experiments.

(B) Serine protease zymography of the sera from 9 ICRP-MDs with non-risk-alleles (A/A) and risk-alleles (T/T) of *PLG* c.478A>T. Pm, human recombinant plasmin.

(C) Plasmin activity levels. Data are representative of three independent experiments.

NS: not significant.

Figure 4. Plasminogen molecules and plasmin activities were lower in colon lesions isolated from ICRP-MDs with risk-alleles

(A) Western blots of the lesions of 8 ICRPs with non-risk-alleles (A/A) and risk-alleles (T/T) of *PLG* c.478A>T. An antibody for plasminogen (the epitope of amino acids 461–810) was used. Intact plasminogen molecules (82 kDa) and many lower molecular weight bands are shown. The relative levels of intact plasminogen molecules are shown on the right. Data are representative of two independent experiments. * $p < 0.05$.
(B) Serine protease zymography of the lesions from 11 ICRP-MDs with non-risk-alleles (A/A) and risk-alleles (T/T) of *PLG* c.478A>T. Pm, human recombinant plasmin.
(C) Plasmin activity levels. Data are representative of three independent experiments. ** $p < 0.01$.

Figure 5. Plasminogen levels were lower in colon lesions isolated from ICRP-MDs with risk-alleles

(A) Representative immunohistochemistry images of lesions of ICRPs with non-risk-alleles (A/A) and risk-alleles (T/T) of *PLG* c.478A>T. An antibody for plasminogen (the epitope of amino acids 461–810) was used.
(B) The signal area of plasminogen molecules in the lesions. ** $p < 0.01$.

Figure 6. Plasminogen molecules were detected in ICRP lesions with risk-alleles

(A) Western blots of the lesions of 8 ICRPs with non-risk-alleles (A/A) and risk-alleles

(T/T) of *PLG* c.478A>T. An antibody specific for amino acids 152–163 of T/T (D159 and Y160) was used. Intact plasminogen molecules (82 kDa) and many low molecular weight plasminogen bands are shown.

(B) Representative immunohistochemistry images of the lesions. An antibody against amino acids 152–163 containing two amino acid substitutions (E159D and N160Y) was used.

(C) Quantification of the colon epithelium in (B) using ImageJ. The percentage of positive areas was calculated from 4 lumen structures. ** $p < 0.01$ (Student's t-test).

Figure 7. MMP-9 cleaves plasminogen molecules with risk-alleles and was induced by the IL-6 amplifier in colon with risk-alleles

(A) Western blots of 293T cells expressing canine MMP12, PLAU, CTSD, MMP3, MMP2, or MMP9 plus plasminogen molecules with non-risk-alleles (A/A) and risk-alleles (T/T). The levels of intact plasminogen molecules with the risk-alleles compared to those without are shown below.

(B) A nonimmune cell line (H4) was stimulated with human IL-6 plus soluble IL-6R and/or TNF- α for 3 h. The mRNA expressions of IL-6 and MMP9 were measured by real-time PCR. Data are representative of three independent experiments, each with four subjects. ** $p < 0.01$.

(C) Representative immunohistochemistry images of the colons of ICRPs with non-risk-alleles (A/A) or risk-alleles (T/T). Antibodies for MMP9, phospho-STAT3, phospho-NF- κ B (phospho-p65), and plasminogen with and/or without risk-alleles were used.

(D) Quantification of the colon epithelium in (C) using ImageJ. The lumen structures

that were positive for phopho-STAT3 and phosphor-NF-κB were selected, and the percentage of areas positive for each molecule was calculated. The mean ± SD from the 6 lumen structures is shown. * p<0.05, ** p<0.01 (Welch test or Student's t-test).

Figure 8. Plasminogen levels in the intestine of patients suffering from ulcerous colitis or Crohn's disease

(A) Representative immunohistochemistry images of human ulcerous colitis, Crohn's disease, and healthy control. Antibodies for MMP9, phopho-STAT3, phosphor-NF-κB, and plasminogen were used. The arrowheads indicate representative examples of cells positive for MMP9, phospho-STAT3, phosphor- NF-κB, and plasminogen.

(B) Quantification of the colon epithelium in (A) using ImageJ. The percentage of positive areas was calculated. The mean ± SD from 12 lumen structures is shown.

** p<0.01, *** p<0.001 (Welch test or Student's t-test). H: healthy control, UC: ulcerous colitis, and CD: Crohn's disease.

Supplementary Figure 1. Representative immunohistochemistry images of the colon lesion of ICRPs with risk-alleles (T/T)

Antibodies for MMP9, phopho-STAT3, phosphor-NF-κB, and plasminogen with and/or without risk-alleles were used.

Supplementary Figure 2. Correlation analysis on the images of the colons of ICRPs with non-risk-alleles (A/A) or risk-alleles (T/T)

The signals positive for MMP9, phopho-STAT3, phosphor-NF-kB (phopho-p65), plasminogen (whole), and plasminogen (aa 152-163) at the same lumen structures

were quantified. The percentages of the positive areas in the images of the colons of ICRP with non-risk-alleles (open circles) and with risk-alleles (gray triangles) are plotted, and the regression lines are depicted in the figures. The Pearson correlation coefficient (r) and p -value (p) are shown on the right of each figure. The levels of MMP9 were positively correlated with those of phospho-p65 or phospho-STAT3, but the levels of plasminogen (whole) were negatively correlated with those of MMP9, phospho-p65, or phospho-STAT3 in the colons of ICRPs with and without the risk-alleles.

Supplementary Figure 3. Correlation analysis on the images of colons of the patients with the human ulcerous colitis (UC) and Crohn's disease (CD), and those of healthy control (HC)

The signals positive for MMP9, phospho-STAT3, phospho-NF- κ B (phospho-p65), and plasminogen (whole) at the same lumen structures were quantified. The percentages of the positive areas in the images of the human ulcerous colitis (gray triangles), Crohn's disease (black squares), and healthy control (open circles) are plotted, and the regression lines are depicted in the figures. The Pearson correlation coefficient (r) and p -value (p) are shown on the right of each figure. The levels of MMP9 were positively correlated with those of phospho-p65 or those of phospho-STAT3, but the levels of plasminogen were negatively correlated with those of MMP9 in the colons of the UC and CD patients and HC.

Figure 1

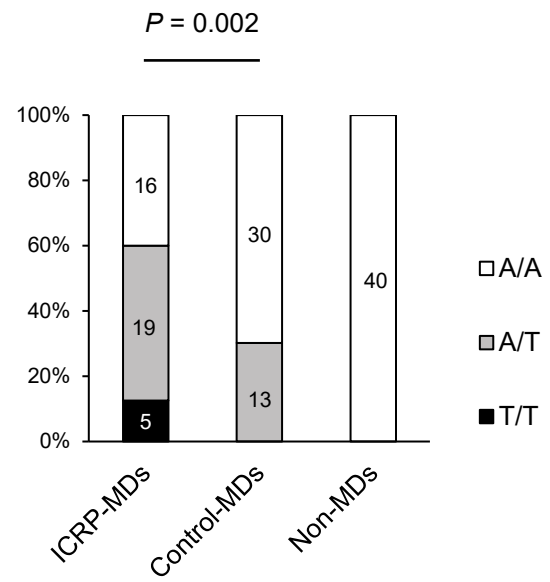


Figure 2

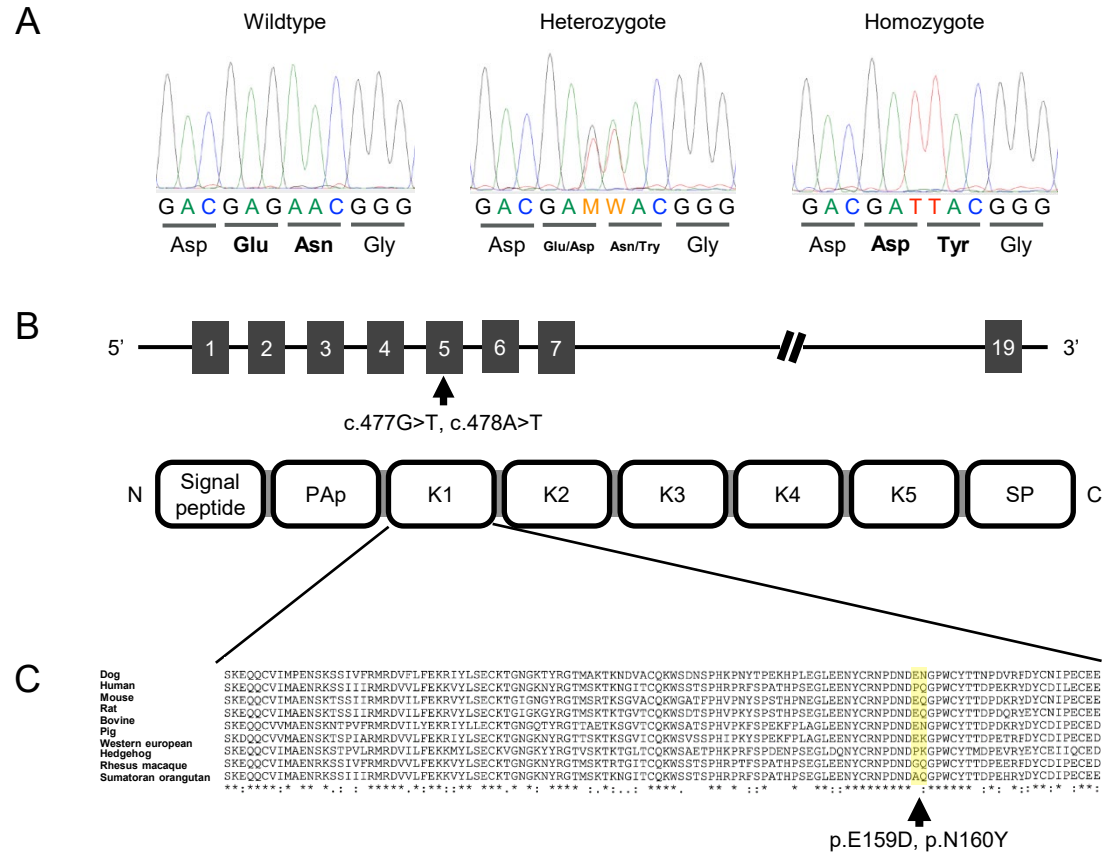


Figure 3

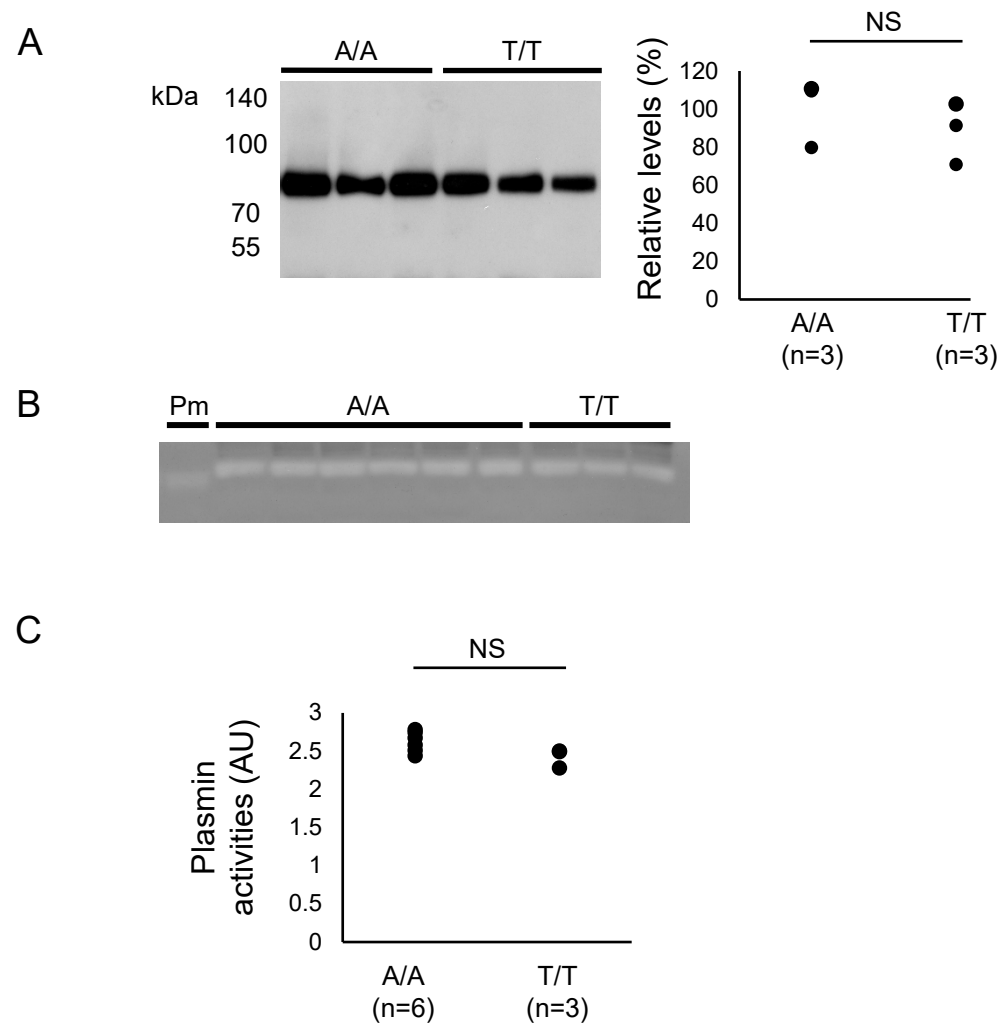


Figure 4

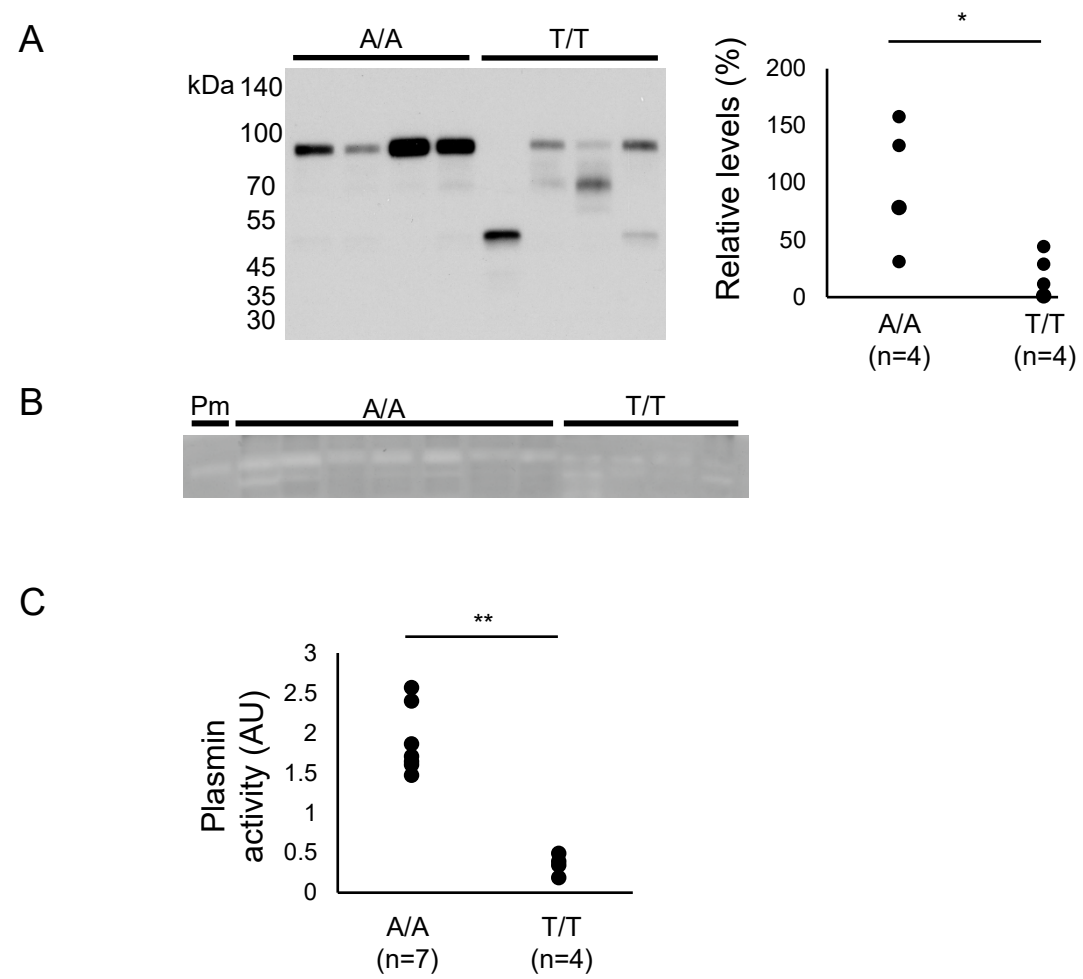


Figure 5

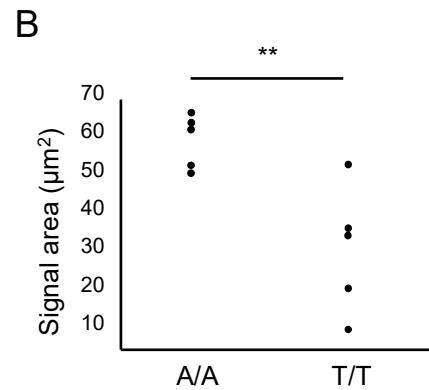
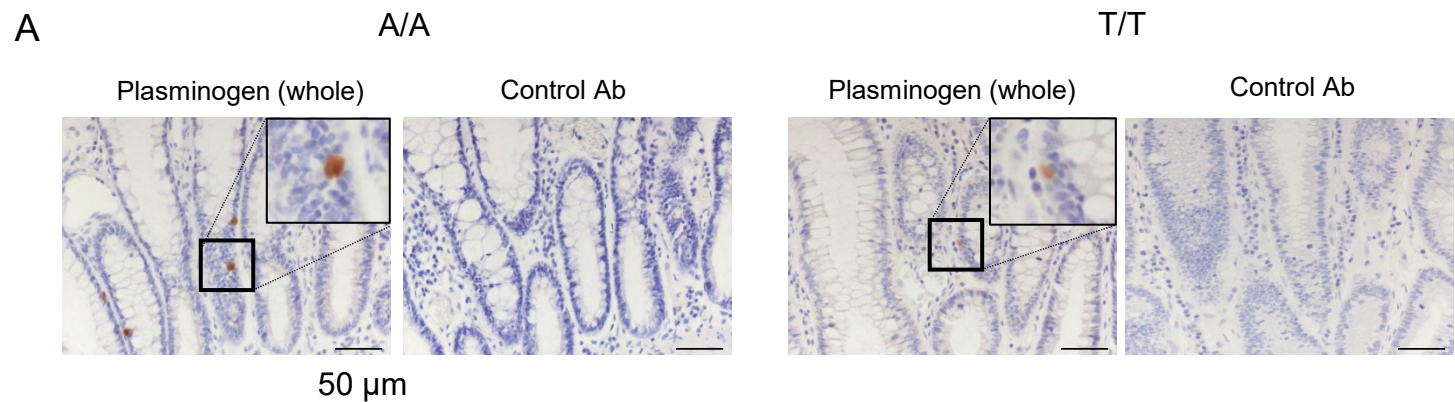


Figure 6

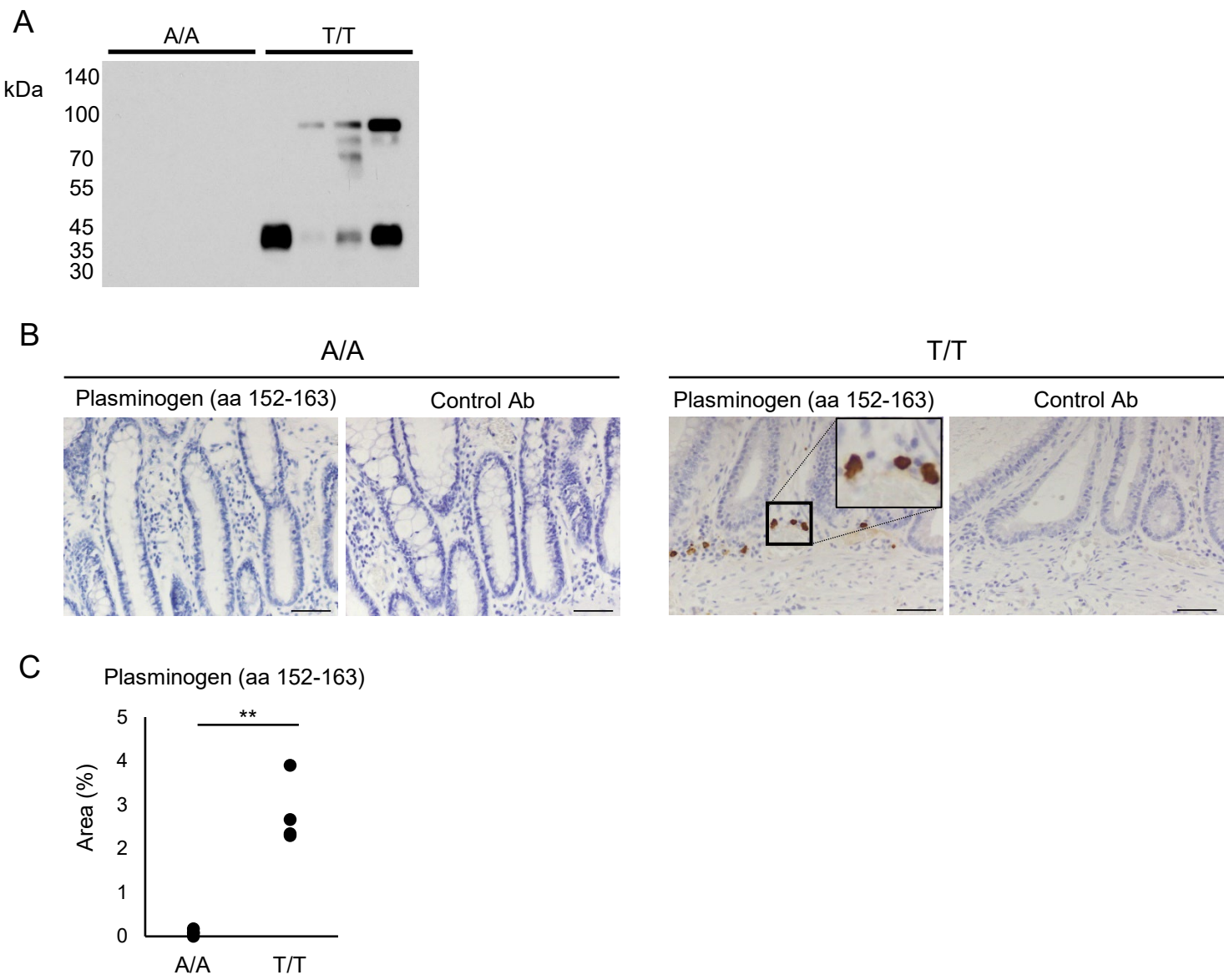


Figure 7

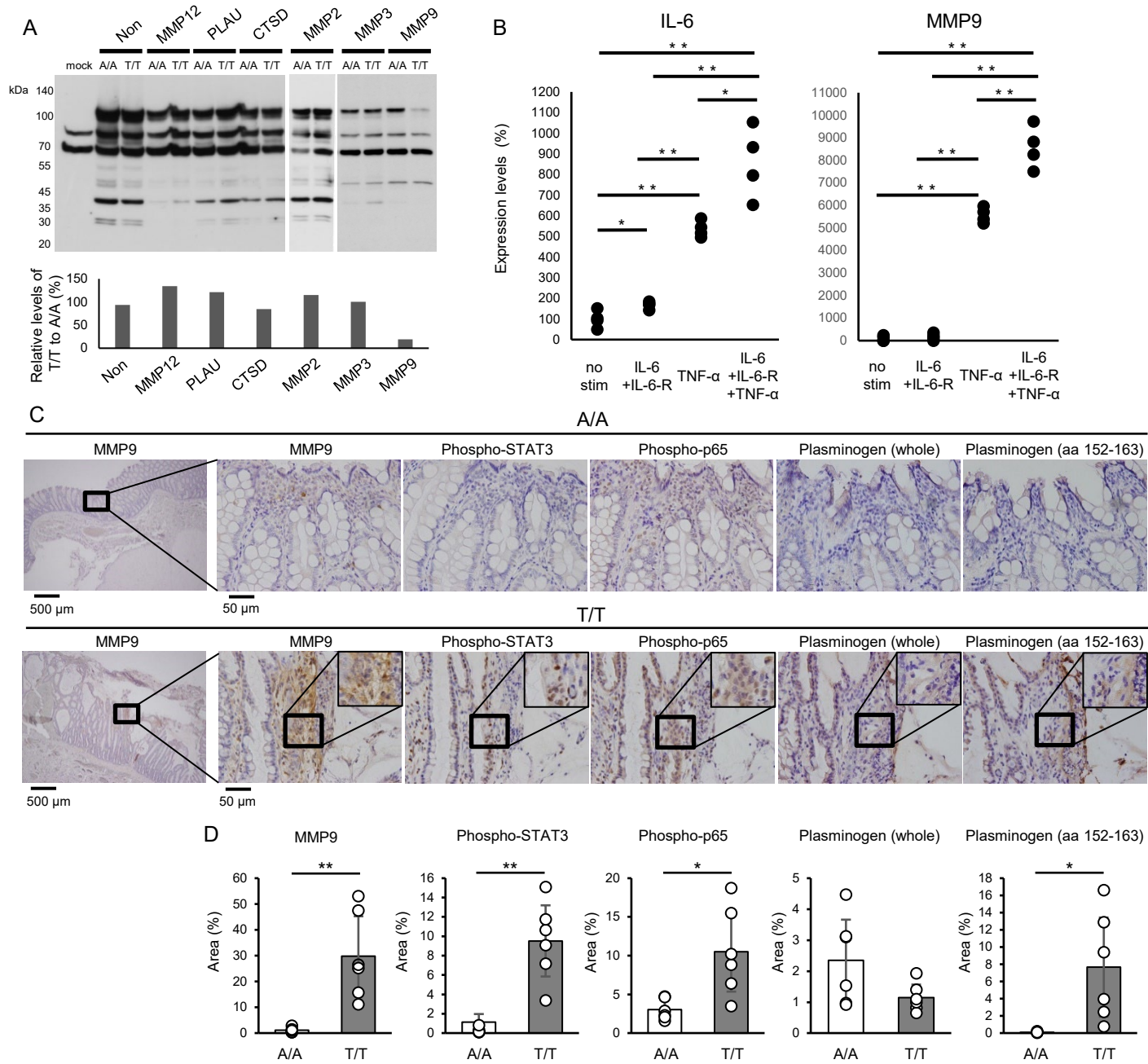
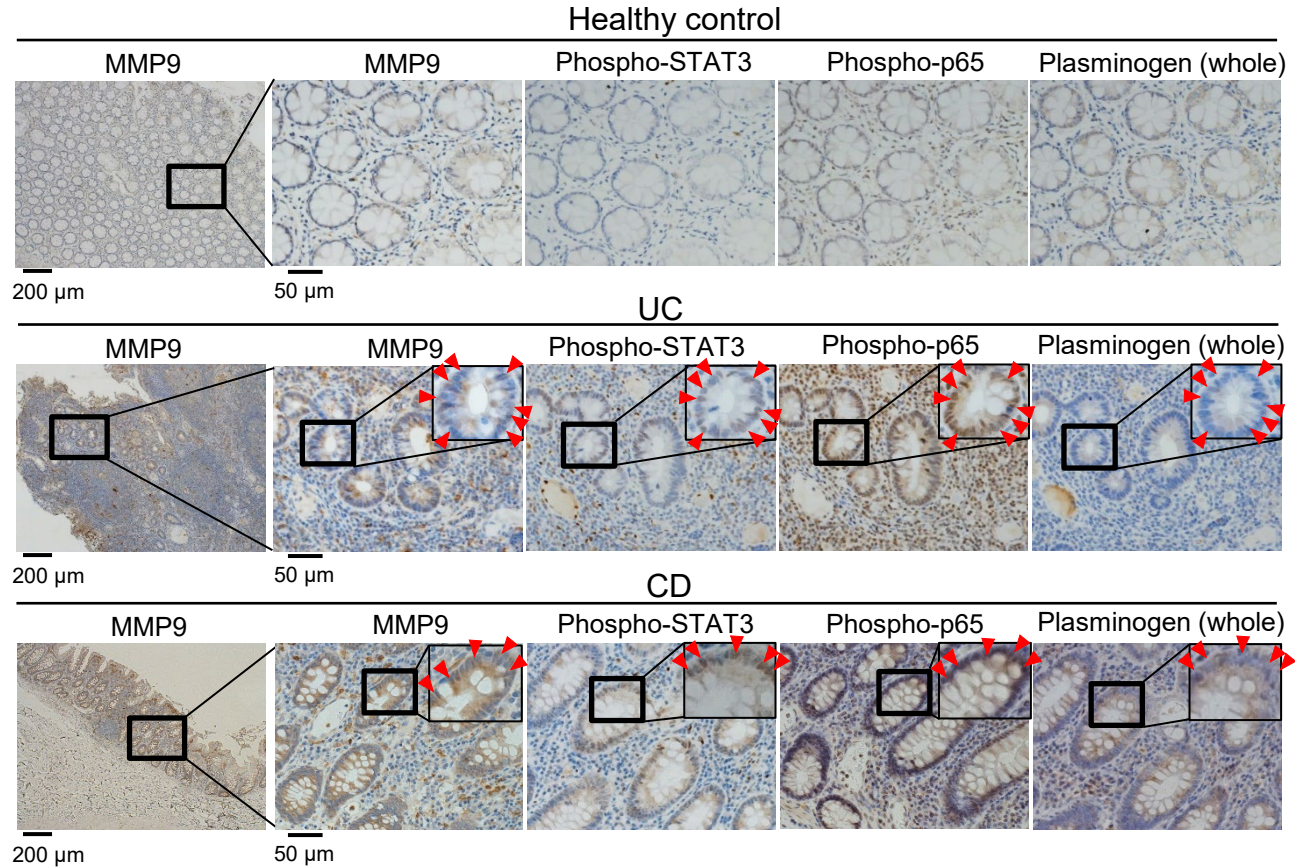


Figure 8

A



B

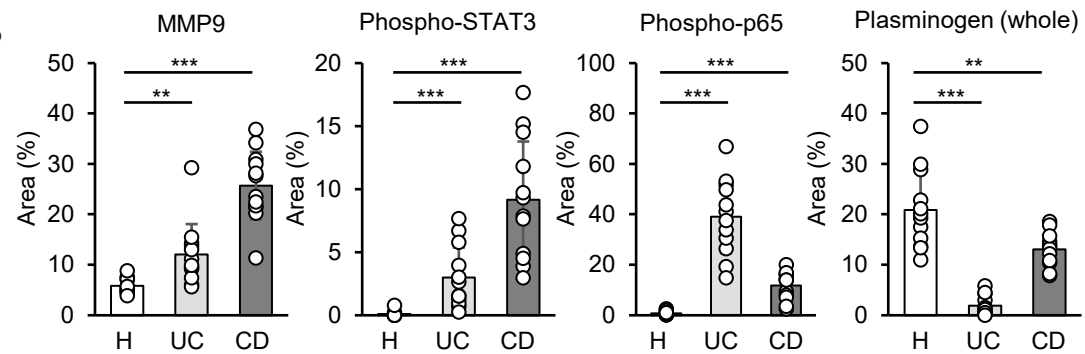


Table 1. Demographic and clinical data of human patients.

Patient	Age	Gender	Race	Diseases	Clinical score
1*	30	Female	Native Japanese	Ulcerous colitis	Severe
2	33	Male	Native Japanese	Ulcerous colitis	Moderate
3	39	Female	Native Japanese	Crohn's disease	Moderate

*Histopathologically normal regions of the colon from the patient 1 were used as the healthy control sample.