The Plasminogen Activator/Plasmin System Is Essential for Development of the Joint Inflammatory Phase of Collagen Type II-Induced Arthritis

Jinan Li,* Anneli Ny,* Göran Leonardsson,*
Kutty Selva Nandakumar,[†] Rikard Holmdahl,[†] and
Tor Ny*

From the Department of Medical Biochemistry and Biophysics,*
Umeå University, Umeå; and the Section for Medical
Inflammation Research,[†] Lund University, Lund, Sweden

The plasminogen activator (PA) system has been proposed to have important roles in rheumatoid arthritis. Here we have used the autoimmune collagen type II (CII)-induced arthritis (CIA) model and mice deficient for urokinase-type PA (uPA) or plasminogen to investigate the role of the PA system for development of arthritis. Our data revealed that uPA-deficient mice have a lower severity and incidence of CIA than wild-type mice. Furthermore, although >80% of wild-type control mice developed CIA, we found that none of the 50 plasminogen-deficient littermates that were tested developed CIA within a 40-day period. Antibody generation after CII immunization as well as the binding of labeled anti-CII antibodies to the surface of cartilage were similar in wild-type and plasminogen-deficient mice. No sign of inflammation was seen when plasminogen-deficient mice were injected with a mixture of monoclonal antibodies against CII. However, after daily injections of human plasminogen, these mice developed arthritis within 5 days. Our finding that infiltration of inflammatory cells into the synovial joints was impaired in plasminogen-deficient mice suggests that uPA and plasminogen are important mediators of joint inflammation. Active plasmin is therefore essential for the induction of pathological inflammatory joint destruction in CIA. (*Am J Pathol* 2005, 166:783–792)

The plasminogen activator (PA) system is a general enzyme system that provides proteolytic activity in many biological processes involving extracellular matrix degradation, tissue remodeling, complement activation, and cell migration.^{1–3} This system has also been suggested

to play an important role in the development of rheumatoid arthritis (RA).^{4–6} The activation of plasminogen to the broad-spectrum protease plasmin is performed by either of the two physiological PAs, tissue-type PA (tPA) or urokinase-type PA (uPA).^{1,2} Activation of the PA system is initiated by the release of PAs from specific cells in response to external signals and results in local proteolytic activity.^{1,2} Because of the high concentration of plasminogen in virtually all tissues, the production of relatively small amounts of PAs can result in high local concentrations of plasmin. By acting in concert with other proteinases, plasmin has also been proposed to play a role in degradation of the extracellular matrix during many physiological and pathological processes such as ovulation,⁷ wound healing,⁸ tumor cell invasion,⁹ angiogenesis,¹⁰ and RA.¹¹

RA is a common human autoimmune disease with a worldwide incidence of ~1%. It is characterized by an erosive inflammatory attack on cartilaginous joints, resulting in synovitis, pannus formation and progression, cartilage and bone destruction, and eventually joint deformity.^{12,13} Both humoral^{13,14} and cellular¹⁵ immune mechanisms are considered to be responsible for the induction of RA. The degradation of the extracellular matrix that takes place during RA is dependent on the action of proteolytic enzymes secreted by both soft and hard tissue cellular elements, as well as by inflammatory cells.^{11,16} Despite significant advances in our understanding of RA, there is still a lack of knowledge regard-

Supported by grants from the Swedish Foundation for Strategic Research (Network for Inflammation Research funding to J.L.), the Swedish Medical Research Council (grant 521-2002-6547), the National Cancer Foundation (grant 03 0549), the Medical Faculty of Umeå University, and the County Council of Västerbotten.

Accepted for publication October 14, 2004.

Present address of A.N.: Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, KU Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

Address reprint requests to Tor Ny, Department of Medical Biochemistry and Biophysics, Umeå University, SE-901 87 Umeå, Sweden. E-mail: tor.ny@medchem.umu.se.

ing the pathogenesis surrounding the initiation and progression of this debilitating disease.

Collagen type II-induced arthritis (CIA) is the most widely used model for RA. CIA is induced in susceptible mouse strains after an intradermal immunization with collagen type II (CII) emulsified in an adjuvant.^{17–20} CIA is a chronic erosive inflammatory disease affecting peripheral joints, and the tissue distribution and histopathology of the destruction process mimic that of RA. CII is the major protein constituent of joint cartilage and the immunization provokes an autoimmune response that attacks the joint. The autoimmune response to CII in CIA is complex and requires MHC molecules, specific T- and B-cell immune responses and their associated cytokines, and several other cellular and biochemical functions. Both T and B cells are essential in the pathogenesis of CIA, but their relative importance in both priming of immune activation and joint destruction are still unclear.²¹ Furthermore, it is well-established that generation of CII-specific antibodies is required in the progression of CIA. Accordingly, transfer of CII-specific monoclonal antibodies induces an acute form of arthritis (CII antibody-induced arthritis model, CAIA).^{22–26} Recent studies on CAIA suggest that both the classical and the alternative pathways of complement activation are involved in the effector phase of arthritis.²⁷

Mice with deficiencies in different components of the PA system provide useful model systems for studying the role of the PA system *in vivo*.^{28–30} Studies of antigen-induced arthritis (AIA) have shown that uPA- and plasminogen-deficient mice have an exacerbated disease severity that correlates with the level of fibrin deposition.³¹ It has therefore been suggested that uPA and plasminogen may play major roles in fibrin removal in the AIA model. However, studies of uPA- and tPA-deficient mice of C57BL/6 genetic background in a CIA model showed that uPA-deficient mice develop only mild CIA, whereas tPA-deficient mice develop a more severe disease as compared to wild-type controls.³² We therefore set out to do an in-depth investigation of the functional roles of the PA system during CIA. In this study we have used uPA- and plasminogen-deficient mice with a CIA susceptible background (DBA/1). Here we show that plasminogen-deficient mice cannot develop CIA, suggesting that the PA system plays an essential role during the development of arthritis. The results also suggest a new therapeutic strategy for treatment of human RA.

Materials and Methods

Animals

uPA-deficient (uPA^{-/-}) mice²⁹ were backcrossed to mice with the C57BL/6 genetic background for seven generations and then crossed to the DBA/1 background for one generation to obtain susceptibility to CIA. Thereafter, the uPA heterozygous (uPA^{+/-}) mice were intercrossed to generate wild-type (uPA^{+/+}), uPA^{+/-}, and uPA^{-/-} mice, all experiments were made on littermates to exclude nonlinked genetic effects. The different uPA ge-

notypes were determined by polymerase chain reaction genotyping.³³ Plasminogen-deficient (plg^{-/-}) mice³⁰ were backcrossed to mice with the C57BL/6 genetic background for seven generations and then crossed to the DBA/1 background for two generations to obtain CIA susceptibility. The plasminogen locus is linked to the MHC region on chromosome 17; therefore, plasminogen heterozygous (plg^{+/-}) mice expressing the MHC II region from the DBA/1 background were selected to ascertain that the mice expressed the CIA-permissive MHC class II H-2 A^q.³⁴ Thereafter, plg^{+/-} mice were further intercrossed to generate wild-type (plg^{+/+}), plg^{+/-}, and plg^{-/-} mice. The plasminogen genotypes of the mice were determined by polymerase chain reaction genotyping and by measuring plasma levels of plasminogen, as described previously.³⁵ For CIA and CAIA experiments, male littermates at the age of 8 to 10 weeks were used. For *in vivo* identification of anti-CII antibody binding to cartilage, 2-day-old neonatal mice were used. All of the mice were selected for positive MHC class II H-2 A^q expression by polymerase chain reaction before the experiment for susceptibility to CIA, as described previously.³⁴ Five plg^{+/+} and five plg^{-/-} mice were also randomly selected and investigated for the expression of MHC class II H-2 A^q expression by fluorescence activated cell-sorting analyses, as described elsewhere.³⁴ The regional ethical committee of Umeå University approved all experimental protocols.

Induction of Collagen Type II-Induced Arthritis (CIA)

Rat CII was prepared from Swarm chondrosarcoma after pepsin digestion.³⁶ CII was dissolved at a concentration of 2 mg/ml in 0.1 mol/L acetic acid and stored at 4°C. CIA was induced by intradermal injection at the base of the tail with 100 μ l of rat CII [100 μ g emulsified 1:1 (v/v) on ice with complete Freund's adjuvant (containing *Mycobacterium butyricum*; Difco, Detroit, MI)]. Twenty-one days after the first immunization, the mice received a boost immunization intradermally at the base of the tail [50 μ l of 50 μ g rat CII emulsified 1:1 (v/v) on ice with incomplete Freund's adjuvant (Difco)].

Monoclonal Antibody Preparation

Monoclonal anti-CII antibodies CIIC1²⁵ and M2139³⁷ have already been described in detail regarding epitope specificity, affinity, autoreactivity, and arthritogenicity. The anti-CII antibody-producing hybridomas for CIIC1 and M2139 were cultured in bovine Ig-free medium (Gibco BRL, Invitrogen AB, Lund, Sweden) and the antibodies were further purified on γ -bind plus affinity gel matrix (Pharmacia, Sweden) as described elsewhere.^{27,36}

Induction of CII Antibody-Induced Arthritis Model (CAIA)

The monoclonal anti-CII antibody cocktail was prepared by mixing 4.5 mg of C1 and 4.5 mg of M2139 to a volume

of 0.4 ml phosphate-buffered saline (PBS). CAIA was induced in mice by intravenous injection of the cocktail twice with a minimum of 3-hour intervals on day 0. On day 5, 50 μg of lipopolysaccharide in 200 μl of PBS were injected intraperitoneally to enhance the response.

Clinical Evaluation of Arthritis

The development of CIA and CAIA was evaluated blindly using a scoring system based on the number of inflamed joints in each paw.³¹ Inflammation was defined by the swelling and redness of the joints. In this scoring system, each inflamed toe or knuckle gives 1 point, whereas an inflamed wrist or ankle gives 5 points, resulting in a score of 0 to 15 (five toes + five knuckles + one wrist/ankle) for each paw and 0 to 60 points for each mouse. Healed joints that are deformed or swollen without redness are not considered in this system.

Quantification of CII-Specific Antibody Titers in Serum by Enzyme-Linked Immunosorbent Assay

Twelve $\text{plg}^{+/+}$ and eleven $\text{plg}^{-/-}$ mice were randomly selected for the quantification of CII-specific antibody titers in serum by enzyme-linked immunosorbent assay. Mice were tail bled at day 40 after CII boost immunization, and the individual sera were stored at -20°C until assayed. Enzyme-linked immunosorbent assay was performed as previously described.³⁸ In brief, 96-well plates were first coated with 50 μl /well PBS containing CII at 10 $\mu\text{g}/\text{ml}$. After washing with Tris-buffered saline (pH 7.4) containing 0.1% Tween 20, the plates were incubated with the diluted sera. They were further incubated with a sheep anti-mouse IgG monoclonal antibody (Jackson ImmunoResearch, West Grove, PA) and the bound IgG was visualized with paranitrophenol as the chromogenic substrate. The amount of CII-specific antibodies in sera was determined by comparing the titration curve of the test serum with the titration curve of a standard consisting of affinity-purified CII-specific antibodies from DBA/1 mice³⁹ with known IgG concentrations.

Identification of the Formation of CII and Anti-CII Immune Complex in Vivo

Biotinylation of anti-CII antibody C1 was performed as previously described.²⁵ Biotinylated anti-CII antibody C1 was injected intraperitoneally (100 $\mu\text{g}/50 \mu\text{l}/\text{mouse}$) into 2-day-old two $\text{plg}^{+/+}$ and two $\text{plg}^{-/-}$ neonatal littermate mice. The mice were sacrificed after 24 hours and the paws were removed and immediately snap-frozen in isopentane that had been prechilled with liquid nitrogen, and embedded in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN). Binding of antibodies *in vivo* was visualized with affinity cytochemical staining using avidin-biotin-peroxidase complexes as described previously.²² As controls, two $\text{plg}^{+/+}$ and two $\text{plg}^{-/-}$ 2-day-old neonatal littermate mice were injected intravenously with PBS.

Morphological Staining of Arthritis

The mice were first sacrificed by decapitation. The wrist and paw joints were removed, fixed in a 4% phosphate-buffered paraformaldehyde solution at 4°C for 24 hours, decalcified in 10% ethylenediaminetetraacetic acid solution for 3 weeks, and then embedded in paraffin. Sections of 8 μm were stained with either hematoxylin-erythrosin or fast green-safranin O.

Immunohistochemical Staining of Macrophages

Mouse paws were decalcified as described for morphology. The immunohistochemical techniques for staining the decalcified paws have been described previously.⁴⁰ Rat anti-mouse CD11b monoclonal antibody was purchased from BD Pharmingen (M1/70, 3 $\mu\text{g}/\text{ml}$; Pharmingen, Stockholm, Sweden) and neutrophils was purchased from Cedarlane (clone 7/4, 3 $\mu\text{g}/\text{ml}$; Cedarlane, Ontario, Canada).

Induction of Arthritis in $\text{plg}^{-/-}$ Mice Supplemented with Human Plasminogen

To restore the plasminogen levels in $\text{plg}^{-/-}$ mice, human plasminogen (10 mg/ml; Biopool, Umeå, Sweden) was injected intravenously (100 $\mu\text{l}/\text{mouse}$) into a group of five $\text{plg}^{-/-}$ mice every 24 hours during the 10-day experimental period. Twelve hours after the first injection, CAIA was induced as described above. As controls, five $\text{plg}^{-/-}$ mice were injected intravenously with sterile PBS. In addition, six $\text{plg}^{+/-}$ and five $\text{plg}^{+/+}$ mice without any intravenous injections were used as untreated controls.

Statistical Analysis

Incidence of arthritis was defined as the proportionate group frequency. Fisher's exact test was used for incidence of arthritis. The Mann-Whitney *U*-test was used for analysis of mean maximum score divided by the number of arthritic mice, mean maximum score divided by the total number of mice, and the day of onset. Antibody levels were analyzed by the two-tailed unpaired *t*-test. $P < 0.05$ was considered to be significant.

Results

Reduced CIA Severity in uPA-Deficient Mice

To examine the role of the PA system during the development of arthritis, CIA was induced in $\text{uPA}^{-/-}$, $\text{uPA}^{+/-}$, and $\text{uPA}^{+/+}$ DBA/1 mice. As shown in Figure 1A, all mice had similar clinical scores during the first 4 days after the boost immunization. However, after day 4, the $\text{uPA}^{+/+}$ mice developed a much more severe arthritis than their $\text{uPA}^{-/-}$ littermates. The difference in both severity and incidence of arthritis between $\text{uPA}^{-/-}$ and $\text{uPA}^{+/+}$ control mice was significant from day 10 onwards ($P < 0.05$, Figure 1). From day 30 and onwards, both the severity

Figure 1. uPA^{-/-} mice have less severe CIA than uPA^{+/+} and uPA^{+/-} littermates. The course of disease is represented by mean arthritis score for all mice in each group (A) and incidence (B) throughout time. The difference in both severity and incidence of arthritis between uPA^{-/-} and uPA^{+/+} control mice was significant from day 10 to day 40.

and incidence of arthritis were also significantly higher in uPA^{+/-} mice than in uPA^{-/-} mice. Throughout the experiment, no significant difference in severity and incidence was seen between the uPA^{+/-} and uPA^{+/+} groups (Figure 1). As shown in Table 1, the uPA^{-/-} mice also showed a significantly delayed disease onset and lower incidence of arthritis as compared to uPA^{+/+} and

uPA^{+/-} mice. Both the uPA^{+/+} and uPA^{+/-} groups also had a significantly higher average maximum clinical score per mouse compared to the uPA^{-/-} group. However, when the mean maximum score was divided by the number of arthritic mice, the uPA^{+/+} control group, but not the uPA^{+/-} group, had a significantly higher clinical score as compared to the uPA^{-/-} group (Table 1). At the end of the experiment 40 days after the boost injection, the paws of the mice were removed for morphological analysis (Figure 3). Paws from uPA^{+/+} and uPA^{-/-} mice that had the same degree of clinical severity showed similar morphology. In arthritic paws from uPA^{+/+} and uPA^{-/-} mice, severe invasion by inflammatory cells, destruction of cartilage and underlying bone, and newly formed cartilage and bone tissues was seen. Taken together, these findings suggest that uPA plays an important role in CIA.

Plasminogen-Deficient Mice Are Resistant to CIA

To study the role of plasminogen/plasmin in the development of arthritis, CIA was induced in plg^{-/-}, plg^{+/-}, and plg^{+/+} mice. As described in Materials and Methods, all experiments were performed with littermates of a mixed but balanced genetic background. All of the mice expressed the CIA-permissive MHC class II H-2 A^q derived from DBA/1. Surprisingly, none of the 50 plg^{-/-} mice tested in the study developed clinical signs of inflammation during the entire period of the experiment (Figure 2). In addition, the plg^{+/-} mice, which have ~50% of the normal serum level of plasminogen (data not shown), developed a lower severity and incidence of arthritis as compared to the plg^{+/+} control group (Table 2, Figure 2). Furthermore, the plg^{+/-} mice had a significantly delayed onset of arthritis as compared to the plg^{+/+} control mice, indicating that induction of CIA is dependent on the level of plasminogen (Table 2). No difference was seen in the maximum score divided by either the total number of mice or only the number of arthritic mice between plg^{+/+} control and plg^{+/-} groups (Table 2), indicating that half of the normal plasminogen level is sufficient to trigger and develop the disease. At the end of the experiment, morphological evaluation revealed that the plg^{-/-} mice had normal joint morphology without inflammation and tissue destruction in the peripheral synovial tissues (Figure 3D). This suggests that the plg^{-/-} mice cannot develop CIA. The plg^{+/-} and plg^{+/+} control mice that were arthritic

Table 1. uPA-Deficient Mice Are Less Prone to CIA

Genotype	Number of mice used	Incidence of arthritis (%) [*]	Day of onset after primary immunization [†]	Mean maximum score (divided by number of arthritic mice) [†]	Mean maximum score (divided by total number of mice) [†]
uPA ^{+/+}	48	85.4 [‡]	35.3 ± 14.9 [‡]	38.2 ± 18.6 [‡]	32.7 ± 22.0 [§]
uPA ^{+/-}	22	90.9 [‡]	35.8 ± 13.5 [‡]	29.8 ± 19.3	27.1 ± 20.4 [§]
uPA ^{-/-}	49	57.1	44.5 ± 17.7	24.3 ± 15.7	14.4 ± 17.0

^{*}The percentage of the number of arthritic mice divided by the total number of mice in each group.

[†]Data are presented as means ± SD.

[‡]*P* < 0.05; [§]*P* < 0.01. uPA^{+/+} and uPA^{+/-} groups were compared with the uPA^{-/-} group.

Figure 2. Plg^{-/-} mice are resistant to CIA and plg^{+/-} mice develop a lower severity of arthritis as compared to the plg^{+/+} control group. The disease course is represented by mean arthritis score for all mice in each group (A) and incidence (B) throughout time.

had similar morphology, with typical signs of arthritis: proliferation of inflammatory cells, formation of pannus, cartilage and bone degradation, and also new bone and cartilage generation (data not shown). Immunohistochemical staining for macrophages and neutrophils showed no infiltration of neutrophils and only a few resting macrophages in joints from plg^{-/-} mice. In contrast, influx of inflammatory cells could be seen in areas of

tissue destruction in the joints of the arthritic plg^{+/+} mice (data not shown).

The Anti-CII Antibody Titers Are Normal in Plasminogen-Deficient Mice

We further investigated whether the inability to develop CIA was because of an impaired humoral immune response to CII.³⁹ Forty days after CII boost injection, sera were randomly collected from 11 plg^{-/-} and 12 plg^{+/+} control mice and the serum IgG antibodies specific for CII were measured by enzyme-linked immunosorbent assay. Although the plg^{+/+} mice had developed the disease and the plg^{-/-} mice were unaffected, the CII-specific IgG antibody levels were comparable in the two genotype groups (Table 2). This result suggests that the deficiency of plasminogen does not affect development of a CII autoimmune response.

Plasminogen-Deficient Mice Are Resistant to CII Antibody-Induced Arthritis (CAIA) but Become Susceptible after Supplementation with Plasminogen

To directly test if plasminogen plays a role in the effector phase of inflammation and tissue destruction, CAIA²⁶ was induced in six plg^{-/-}, seven plg^{+/-}, and five plg^{+/+} littermate mice. As shown in Figure 4, all of the plg^{+/+} and plg^{+/-} mice developed arthritis within 5 days after injection of anti-CII antibody cocktail. The plg^{-/-} mice, however, did not show any signs of inflammation throughout the 46-day experiment period. The arthritis that the plg^{+/+} mice developed was more severe than the arthritis the plg^{+/-} mice developed. The severity of arthritis in plg^{+/+} and plg^{+/-} mice reached a peak at approximately day 10 and continued to be high for an additional 20 days. At the end of the experiment (day 46), both plg^{+/+} and plg^{+/-} mice had essentially recovered from arthritis. Because injection of anti-CII antibody cocktail directly triggers an inflammation and tissue destruction response, this result suggests that plasminogen has a role to play during the effector stage of arthritis. Morphological analysis of paws that were collected from arthritic mice at day 10 after antibody injection revealed that plg^{+/+} and plg^{+/-} mice had active inflammation and tissue destruction, but there were no signs of inflammation or tissue destruction in the plg^{-/-} mice (data not shown).

Table 2. Plasminogen-Deficient Mice Are Resistant to CIA

Genotype	Number of mice used	Incidence of arthritis (%) [*]	Day of onset after primary immunization [†]	Mean maximum score (divided by number of arthritic mice) [‡]	Mean maximum score (divided by total number of mice) [‡]	IgG (μg/ml) [‡]
Plg ^{+/+}	30	83.3	37.6 ± 15.0	39.4 ± 18.5	32.8 ± 22.5	356.8 ± 369.8
Plg ^{+/-}	19	68.4	45.8 ± 14.2 [‡]	37.4 ± 19.3	27.6 ± 23.6	ND
Plg ^{-/-}	50	0	0	0	0	273.1 ± 258.9

^{*}The percentage of the number of arthritic mice divided by the total number of mice in each group.

[†]Data are presented as means ± SD.

[‡]P < 0.05. Plg^{+/-} group was compared with plg^{+/+} group.

ND, not determined.

Figure 3. Representative morphological sections of paws from mice 40 days after CIA boost injection. **A:** Joint section from a wild-type unaffected mouse showing the intact cartilage (red), bone (blue), and normal synovium without inflammation. **B:** Joint section from a wild-type mouse with CIA. Influx of inflammatory cells, newly formed pannus, and extensive degradation and reformation of cartilage and bone erosion are shown. **C:** Joint section from a $uPA^{-/-}$ mouse with CIA, showing similar morphology to that of the wild-type mice with CIA. **D:** Joint section from a $plg^{-/-}$ mouse without CIA, showing normal morphology as compared to the wild-type unaffected mice. No inflammation or tissue destruction was observed. The sections were stained with fast green-safranin O. Original magnifications, $\times 100$.

To confirm that the inability to develop arthritis in $plg^{-/-}$ mice is because of plasminogen deficiency, 100 μ l of human plasminogen (10 mg/ml) or 100 μ l of PBS

were injected into $plg^{-/-}$ mice at 24-hour intervals.⁴¹ Twelve hours after the first administration, CAIA was induced in these mice, and also in $plg^{+/+}$ and $plg^{+/-}$ mice that served as controls (Table 3). During the 10-day

Figure 4. $Plg^{-/-}$ mice are resistant to CAIA and $plg^{+/-}$ mice develop a lower severity of arthritis as compared to $plg^{+/+}$ control mice. The disease course is represented by mean arthritis score for all mice in each group throughout time.

Table 3. Susceptibility of Plasminogen-Deficient Mice to CAIA after Supplementation with Plasminogen

Group	Incidence*	Mean maximum score (divided by number of arthritic mice) [†]
$Plg^{+/+}$	5/5	32.2 ± 9.7
$Plg^{+/-}$	6/6	28.7 ± 21.7
$Plg^{-/-}$ injected with PBS	0/5	0
$Plg^{-/-}$ supplemented with human plasminogen	4/5	10.8 ± 6.7

*Number of arthritic mice divided by the total number of mice in each group.

[†]Data are presented as means $\pm$ SD.

Figure 5. Representative examples of cytochemical staining of joints from neonatal $plg^{+/+}$ (A) and $plg^{-/-}$ (B) mice 24 hours after an intraperitoneal injection of biotinylated syngeneic mouse monoclonal anti-CII antibodies. The sections were stained by direct application of avidin-biotin complexes. Original magnifications, $\times 200$.

experimental period, four of the five $plg^{-/-}$ mice supplemented with plasminogen, as well as all six $plg^{+/-}$ mice and five $plg^{+/+}$ mice, developed joint inflammation within 5 days of antibody injection. In contrast, none of the five PBS-treated $plg^{-/-}$ mice developed any signs of inflammation. Morphological analysis of paws that were collected from arthritic mice at the end of the experiment revealed that $plg^{-/-}$ mice supplemented with plasminogen had active inflammation and tissue destruction in the joints, but there were no signs of inflammation or tissue destruction in PBS-treated $plg^{-/-}$ mice (data not shown). These results confirm that the inability of $plg^{-/-}$ mice to develop arthritis is because of plasminogen deficiency.

Anti-CII Antibodies Normally Bind to Cartilage Surface in Plasminogen-Deficient Mice in Vivo

The above findings show that plasminogen plays an essential role during the effector stage of CIA. Immune recognition and response toward specific cartilage structures is critical for the initiation and development of CIA.⁴² We therefore investigated whether anti-CII antibodies are accessible to CII epitopes on the cartilage of $plg^{-/-}$ mice *in vivo*. This investigation was performed by injecting biotinylated syngeneic mouse monoclonal anti-CII antibodies that are specific for some of the major epitopes on CII into neonatal $plg^{+/+}$ and $plg^{-/-}$ mice.^{22,25,26} As shown in Figure 5, the anti-CII antibodies bound to cartilage sur-

faces facing the joint space and endochondrial spaces as well as cartilage facing the bone marrow in $plg^{-/-}$ mice and in $plg^{+/-}$ control mice. This indicates that the CII epitopes are not masked in $plg^{-/-}$ mice *in vivo* and that the formation of CII and anti-CII immune complex in the cartilage of $plg^{-/-}$ mice is normal *in vivo*.

Discussion

Our studies of $uPA^{-/-}$ mice with a DBA/1 background reveal that mice lacking uPA develop a less severe arthritis with lower incidence, and that the onset of the disease is delayed as compared to $uPA^{+/+}$ control mice. These results are consistent with a previous study in a CIA model using $uPA^{-/-}$ mice with a C57BL/6 background.³² These mice express H-2 A^b and are less permissible to CIA, and develop a disease that is less well characterized than the disease that the H-2 A^d-expressing mice used here. Most strikingly, we also found that whereas $>83\%$ of $plg^{+/+}$ control mice developed severe CIA, none of 50 $plg^{-/-}$ mice that were tested developed CIA within the 40-day experimental period. Furthermore, $plg^{+/+}$ mice injected with monoclonal antibodies to CII developed arthritis within 5 days, whereas no sign of inflammation could be seen in $plg^{-/-}$ mice. After daily injections with human plasminogen, however, the $plg^{-/-}$ mice also developed arthritis within 5 days. Although the generation of anti-CII antibodies and antibody binding were comparable in both $plg^{+/+}$ and $plg^{-/-}$ mice, no infiltrations of inflammatory cells into the synovial joints could be detected in the $plg^{-/-}$ mice. These results indicate that the formation of active plasmin plays an essential role in the pathogenesis of CIA, most likely in a step between the binding of arthritogenic antibodies on joint cartilage and infiltration of inflammatory cells. We propose that activation of plasminogen to active plasmin by uPA can lead to the induction of the pathogenic joint inflammation seen in CIA.

As in RA, the pathogeneses in arthritis models are still quite complex and heterogeneous. CIA is induced by an intradermal immunization with CII emulsified in an adjuvant, and it has been widely used as a mouse model for RA.¹⁷⁻²⁰ In CIA both T and B cells are needed and it is believed that the major effector mechanism is mediated by arthritogenic antibodies. After the injection of rat CII, antigen-presenting cells take up the antigen and subsequently activate the humoral and cellular immune responses in the draining lymph nodes. The activated B cells produce anti-CII antibodies that circulate and bind to the articular CII. The anti CII antibodies bind to cartilage and activates the complement system as well as inflammatory cells such as neutrophils and macrophages,²¹ which can lead to induction of arthritis also in the absence of T and B cells.⁴³ Previous studies have suggested that the anti-CII antibodies undergo several steps toward triggering of arthritis including the involvement of both the classical and alternative complement pathways^{27,44} and Fc receptors.^{45,46} In this study, the CII-specific antibody titers were comparable in $plg^{+/+}$ and $plg^{-/-}$ mice, suggesting that antibody production is

not impaired in $plg^{-/-}$ mice. However, our finding that $plg^{-/-}$ mice were resistant to both CIA and CAIA, but became susceptible to CAIA after plasminogen supplementation, indicating that plasmin plays an essential role during an antibody and complement-dependent but T cell-independent effector stage of CIA.

Joint destruction in RA has been attributed to matrix-degrading enzymes such as matrix metalloproteinases and serine proteases.^{47,48} Among the serine proteases, the PA/plasmin system is of unique interest because of its capacity to degrade a variety of extracellular matrix proteins, and its ability to activate latent forms of matrix metalloproteinases.^{49,50} The cartilage structure recognized by the anti-CII antibodies is of importance for development of arthritis.^{51,52} One possibility could therefore be that plasmin plays a direct or indirect role in cartilage degradation that could lead to an enhanced exposure of CII epitopes and facilitate the formation of CII and anti-CII immune complex. However, as shown in Figure 5, the binding of anti-CII antibodies that are specific for major epitopes on CII bind equally well to cartilage surfaces of $plg^{-/-}$ mice as to $plg^{+/+}$ control mice that develop CIA. These findings, together with the fact that there was a complete absence of inflammatory cell infiltration and tissue destruction in the $plg^{-/-}$ mice after induction of CIA (Figure 3), suggest that plasmin plays an essential role in events downstream of the binding of arthritogenic antibodies to cartilage, possibly the activation of the complement system.

Previous studies have shown that plasmin can activate the complement system *in vitro*.^{3,53,54} However, the *in vivo* significance of this finding has not been demonstrated. Furthermore, it has not been demonstrated whether plasmin activation of complement system plays any role in the development of arthritis. Recent studies in mice deficient in members of the complement system have shown that both alternative and classical pathways of complement activation are important in the development of arthritis in CIA as well as in CAIA.^{27,55–57} C5-deficient mice have normal cellular and humoral immune responses to native CII, but these mice are highly resistant to the induction of CIA.^{56,58} In addition, C5a receptor-deficient mice are completely protected from the development of CAIA, whereas deletion of the C3a receptor appears to have no substantial effect on disease development of CAIA.⁵⁵ In another study, control mice immunized with bovine CII emulsified in complete Freund's adjuvant developed severe arthritis and high CII-specific antibody titers. In contrast, the C3-deficient and factor B-deficient mice were highly resistant to CIA and displayed decreased CII-specific antibody response.⁵⁷ Similar results were obtained in C3- and factor B-deficient DBA/1J mice when well-defined monoclonal IgG2b and IgG2a antibodies to CII were used to induce CAIA. Whereas control DBA/1J mice developed CAIA very rapidly with a 100% incidence and a peak on days 7 to 10, only 75% of C3-deficient mice developed arthritis. In addition, the clinical severity was very mild and the onset was delayed. Severity of arthritis in factor B-deficient mice ranked intermediate in comparison with C3-deficient and control mice with an incidence of 100%.²⁷ These findings are consistent with

our results in $plg^{-/-}$ mice, provided that plasmin plays an essential role in complement activation. Our data therefore suggest that plasmin plays an essential role for the induction of CIA and CAIA, likely in the activation of the complement system. However, despite that plasmin can activate complement *in vitro*, the direct involvement of plasmin in complement activation in arthritis remains to be demonstrated.

Previous studies on AIA, in which methylated bovine serum albumin (mBSA) was used as the antigen, have suggested a protective role for plasmin and uPA in arthritis.³¹ This finding contrasts markedly with our present findings using the CIA and CAIA models. The authors suggested that the exacerbated severity of AIA in $plg^{-/-}$ and $uPA^{-/-}$ mice as compared to wild-type controls is dependent on the persistence of deposited fibrin during the phase of joint inflammation and joint destruction.³¹ CIA is a self-perpetuating arthritis model triggered by autoimmune responses in which the arthritis inflammation is not only dependent on an autoimmune response consisting of arthritogenic antibodies, but also other inflammatory components such as T cells, macrophages, and fibroblasts. In CIA, the fibrin deposition is predominant during the first few days after the inflammation starts.¹² Thereafter, fibrin is eliminated from the joints and tissue degradation starts and predominates during the remaining stages of disease.¹² In AIA, immunization with mBSA starts an adaptive immunity against mBSA. However, the inducing agent mBSA is not a self-protein, and the arthritis induced is a local reaction dependent on the intra-articular injection of mBSA. AIA therefore has a different pathogenesis from CIA. Furthermore, in injecting mBSA into knee joints to enable local binding of mBSA to cartilage, trauma has to be performed. This trauma, however, will also initiate an inflammatory wound-healing-like process in which the innate immune response against trauma is involved. Therefore, during AIA, both adaptive immunity against mBSA and innate immunity against trauma are involved. Thus, the importance of trauma must be taken into account in the pathogenesis of AIA. Extravascular fibrin deposition is an important event in disease states characterized by inflammation and tissue repair. Fibrin deposition in the joints may have deleterious roles such as impeding normal nutrition to the joint tissues leading to hypoxia and acidosis, serving as a provisional matrix onto which cells can adhere and migrate, and enhance the local expression of the proinflammatory cytokines.³¹ Persistent deposition of fibrin has been observed in traumas in $plg^{-/-}$ mice.⁸ In this respect, the fibrin deposition in $plg^{-/-}$ mice in AIA may be a consequence of inflammation associated with trauma instead of being the reason for the inflammation of arthritis, or both.

Our morphological and immunohistochemical studies showed that there was no inflammatory cell infiltration in $plg^{-/-}$ mice after the induction of CIA (Figure 3 and data not shown). Although plasmin has been suggested to play an important role in inflammatory cell migration, our studies on the healing of tympanic membrane perforations (J. Li, P.-O. Eriksson, A. Hansson, S. Hellstrom, T. Ny, submitted for publication) as well as other studies^{8,59–61} have suggested that inflammatory cell migration is in

general not compromised in $plg^{-/-}$ mice. For several reasons we therefore find it unlikely that the essential role of plasmin in CIA found in this study is related to inflammatory cell migration. Rather it appears that plasmin plays an important role in the activation of inflammatory response.

In summary, we have found that the PA/plasmin system plays an essential role in the development of CIA. In the pathway of disease development, plasmin seems to be associated with an event after the binding of arthritogenic antibodies to the cartilage surface, possibly in the activation of the complement system. Given the essential role that plasmin seems to play in the pathway leading to CIA, the present data suggest new therapeutic strategies for the treatment of RA and other autoimmune inflammatory disorders in humans.

References

1. Saksela O, Rifkin DB: Cell-associated plasminogen activation: regulation and physiological functions. *Annu Rev Cell Biol* 1988, 4:93–126
2. Collen D: The plasminogen (fibrinolytic) system. *Thromb Haemost* 1999, 82:259–270
3. Schaiff WT, Eisenberg PR: Direct induction of complement activation by pharmacologic activation of plasminogen. *Coron Artery Dis* 1997, 8:9–18
4. Belcher C, Fawthrop F, Bunning R, Doherty M: Plasminogen activators and their inhibitors in synovial fluids from normal, osteoarthritis, and rheumatoid arthritis knees. *Ann Rheum Dis* 1996, 55:230–236
5. Kummer JA, Abbink JJ, de Boer JP, Roem D, Nieuwenhuys EJ, Kamp AM, Swaak TJ, Hack CE: Analysis of intraarticular fibrinolytic pathways in patients with inflammatory and noninflammatory joint diseases. *Arthritis Rheum* 1992, 35:884–893
6. Saxne T, Lecander I, Geborek P: Plasminogen activators and plasminogen activator inhibitors in synovial fluid. Difference between inflammatory joint disorders and osteoarthritis. *J Rheumatol* 1993, 20:91–96
7. Ny T, Peng XR, Ohlsson M: Hormonal regulation of the fibrinolytic components in the ovary. *Thromb Res* 1993, 71:1–45
8. Romer J, Bugge TH, Pyke C, Lund LR, Flick MJ, Degen JL, Dano K: Impaired wound healing in mice with a disrupted plasminogen gene. *Nat Med* 1996, 2:287–292
9. Andreasen PA, Kjoller L, Christensen L, Duffy MJ: The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 1997, 72:1–22
10. Pepper MS, Sappino AP, Stocklin R, Montesano R, Orci L, Vassalli JD: Upregulation of urokinase receptor expression on migrating endothelial cells. *J Cell Biol* 1993, 122:673–684
11. Del Rosso M, Fibbi G, Matucci CM: The urokinase-type plasminogen activator system and inflammatory joint diseases. *Clin Exp Rheumatol* 1999, 17:485–498
12. Caulfield JP, Hein A, Dynesius-Trentham R, Trentham DE: Morphologic demonstration of two stages in the development of type II collagen-induced arthritis. *Lab Invest* 1982, 46:321–343
13. Feldmann M, Brennan FM, Maini RN: Rheumatoid arthritis. *Cell* 1996, 85:307–310
14. Edwards JC, Cambridge G: Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes. *Rheumatology (Oxford)* 2001, 40:205–211
15. Sekine T, Kato T, Masuko-Hongo K, Nakamura H, Yoshino S, Nishioka K, Yamamoto K: Type II collagen is a target antigen of clonally expanded T cells in the synovium of patients with rheumatoid arthritis. *Ann Rheum Dis* 1999, 58:446–450
16. Cawston T: Matrix metalloproteinases and TIMPs: properties and implications for the rheumatic diseases. *Mol Med Today* 1998, 4:130–137
17. Holmdahl R, Karlsson M, Andersson ME, Rask L, Andersson L: Localization of a critical restriction site on the I-A beta chain that determines susceptibility to collagen-induced arthritis in mice. *Proc Natl Acad Sci USA* 1989, 86:9475–9479
18. Myers LK, Rosloniec EF, Cremer MA, Kang AH: Collagen-induced arthritis, an animal model of autoimmunity. *Life Sci* 1997, 61:1861–1878
19. Trentham DE, Townes AS, Kang AH: Autoimmunity to type II collagen: an experimental model of arthritis. *J Exp Med* 1977, 146:857–868
20. Holmdahl R, Andersson M, Enander I, Goldschmidt T, Jansson L, Larsson P, Mo J, Nordling C, Klareskog L: Nature of the type II collagen autoimmunity in mice susceptible to collagen-induced arthritis. *Int Rev Immunol* 1988, 4:49–64
21. Holmdahl R, Bockermann R, Backlund J, Yamada H: The molecular pathogenesis of collagen-induced arthritis in mice—a model for rheumatoid arthritis. *Ageing Res Rev* 2002, 1:135–147
22. Holmdahl R, Mo JA, Jonsson R, Karlstrom K, Scheynius A: Multiple epitopes on cartilage type II collagen are accessible for antibody binding in vivo. *Autoimmunity* 1991, 10:27–34
23. Terato K, Harper DS, Griffiths MM, Hastly DL, Ye XJ, Cremer MA, Seyer JM: Collagen-induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity* 1995, 22:137–147
24. Holmdahl R, Jansson L, Larsson A, Jonsson R: Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen autoantibodies. *Scand J Immunol* 1990, 31:147–157
25. Holmdahl R, Rubin K, Klareskog L, Larsson E, Wigzell H: Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II collagen antibodies. *Arthritis Rheum* 1986, 29:400–410
26. Nandakumar KS, Svensson L, Holmdahl R: Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *Am J Pathol* 2003, 163:1827–1837
27. Hietala MA, Nandakumar KS, Persson L, Fahlen S, Holmdahl R, Pekna M: Complement activation by both classical and alternative pathways is critical for the effector phase of arthritis. *Eur J Immunol* 2004, 34:1208–1216
28. Bugge TH, Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K, Degen JL: The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. *J Biol Chem* 1995, 270:16886–16894
29. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC: Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* 1994, 368:419–424
30. Ploplis VA, Carmeliet P, Vazirzadeh S, Van Vlaenderen I, Moons L, Plow EF, Collen D: Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. *Circulation* 1995, 92:2585–2593
31. Bussio N, Peclat V, van Ness K, Kolodziejczyk E, Degen J, Bugge T, So A: Exacerbation of antigen-induced arthritis in urokinase-deficient mice. *J Clin Invest* 1998, 102:41–50
32. Cook AD, Braine EL, Campbell IK, Hamilton JA: Differing roles for urokinase and tissue-type plasminogen activator in collagen-induced arthritis. *Am J Pathol* 2002, 160:917–926
33. Ny A, Nordstrom L, Carmeliet P, Ny T: Studies of mice lacking plasminogen activator gene function suggest that plasmin production prior to ovulation exceeds the amount needed for optimal ovulation efficiency. *Eur J Biochem* 1997, 244:487–493
34. Brunsberg U, Gustafsson K, Jansson L, Michaelsson E, Ahrlund-Richter L, Pettersson S, Mattsson R, Holmdahl R: Expression of a transgenic class II Ab gene confers susceptibility to collagen-induced arthritis. *Eur J Immunol* 1994, 24:1698–1702
35. Ny A, Leonardsson G, Hagglund AC, Hagglof P, Ploplis VA, Carmeliet P, Ny T: Ovulation in plasminogen-deficient mice. *Endocrinology* 1999, 140:5030–5035
36. Andersson M, Holmdahl R: Analysis of type II collagen-reactive T cells in the mouse. I. Different regulation of autoreactive vs non-autoreactive anti-type II collagen T cells in the DBA/1 mouse. *Eur J Immunol* 1990, 20:1061–1066
37. Mo JA, Holmdahl R: The B cell response to autologous type II collagen: biased V gene repertoire with V gene sharing and epitope shift. *J Immunol* 1996, 157:2440–2448

38. Corthay A, Johansson A, Vestberg M, Holmdahl R: Collagen-induced arthritis development requires alpha beta T cells but not gamma delta T cells: studies with T cell-deficient (TCR mutant) mice. *Int Immunol* 1999, 11:1065-1073
39. Holmdahl R, Klareskog L, Andersson M, Hansen C: High antibody response to autologous type II collagen is restricted to H-2q. *Immunogenetics* 1986, 24:84-89
40. Holmdahl R, Jonsson R, Larsson P, Klareskog L: Early appearance of activated CD4+ T lymphocytes and class II antigen-expressing cells in joints of DBA/1 mice immunized with type II collagen. *Lab Invest* 1988, 58:53-60
41. Lijnen HR, Carmeliet P, Bouche A, Moons L, Ploplis VA, Plow EF, Collen D: Restoration of thrombolytic potential in plasminogen-deficient mice by bolus administration of plasminogen. *Blood* 1996, 88:870-876
42. Holmdahl R, Andersson M, Goldschmidt TJ, Gustafsson K, Jansson L, Mo JA: Type II collagen autoimmunity in animals and provocations leading to arthritis. *Immunol Rev* 1990, 118:193-232
43. Nandakumar KS, Svensson L, Holmdahl R: Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *Am J Pathol* 2003, 163:1827-1837
44. Watson WC, Brown PS, Pitcock JA, Townes AS: Passive transfer studies with type II collagen antibody in B10.D2/old and new line and C57Bl/6 normal and beige (Chediak-Higashi) strains: evidence of important roles for C5 and multiple inflammatory cell types in the development of erosive arthritis. *Arthritis Rheum* 1987, 30:460-465
45. Diaz DS, Andren M, Martinsson P, Verbeek JS, Kleinau S: Expression of FcgammaRIII is required for development of collagen-induced arthritis. *Eur J Immunol* 2002, 32:2915-2922
46. Nandakumar KS, Andren M, Martinsson P, Bajtner E, Hellstrom S, Holmdahl R, Kleinau S: Induction of arthritis by single monoclonal IgG anti-collagen type II antibodies and enhancement of arthritis in mice lacking inhibitory FcgammaRIIB. *Eur J Immunol* 2003, 33:2269-2277
47. Cunnane G, Hummel KM, Muller-Ladner U, Gay RE, Gay S: Mechanism of joint destruction in rheumatoid arthritis. *Arch Immunol Ther Exp (Warsz)* 1998, 46:1-7
48. van der Laan WH, Pap T, Ronday HK, Grimbergen JM, Huisman LG, TeKoppele JM, Breedveld FC, Gay RE, Gay S, Huizinga TW, Verheijen JH, Quax PH: Cartilage degradation and invasion by rheumatoid synovial fibroblasts is inhibited by gene transfer of a cell surface-targeted plasmin inhibitor. *Arthritis Rheum* 2000, 43:1710-1718
49. Werb Z, Mainardi CL, Vater CA, Harris Jr ED: Endogenous activation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. *N Engl J Med* 1977, 296:1017-1023
50. Medcalf RL, Hamilton JA: Human synovial fibroblasts produce urokinase-type plasminogen activator. *Arthritis Rheum* 1986, 29:1397-1401
51. Schulte S, Unger C, Mo JA, Wendler O, Bauer E, Frischholz S, von der MK, Kalden JR, Holmdahl R, Burkhardt H: Arthritis-related B cell epitopes in collagen II are conformation-dependent and sterically privileged in accessible sites of cartilage collagen fibrils. *J Biol Chem* 1998, 273:1551-1561
52. Burkhardt H, Koller T, Engstrom A, Nandakumar KS, Turnay J, Kraetsch HG, Kalden JR, Holmdahl R: Epitope-specific recognition of type II collagen by rheumatoid arthritis antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse. *Arthritis Rheum* 2002, 46:2339-2348
53. Bode AP, Miller DT, Newman SL, Castellani WJ, Norris HT: Plasmin activity and complement activation during storage of citrated platelet concentrates. *J Lab Clin Med* 1989, 113:94-102
54. Ward PA: A plasmin-split fragment of C'3 as a new chemotactic factor. *J Exp Med* 1967, 126:189-206
55. Grant EP, Picarella D, Burwell T, Delaney T, Croci A, Avitahl N, Humbles AA, Gutierrez-Ramos JC, Briskin M, Gerard C, Coyle AJ: Essential role for the C5a receptor in regulating the effector phase of synovial infiltration and joint destruction in experimental arthritis. *J Exp Med* 2002, 196:1461-1471
56. Wang Y, Kristan J, Hao L, Lenkoski CS, Shen Y, Matis LA: A role for complement in antibody-mediated inflammation: C5-deficient DBA/1 mice are resistant to collagen-induced arthritis. *J Immunol* 2000, 164:4340-4347
57. Hietala MA, Jonsson IM, Tarkowski A, Kleinau S, Pekna M: Complement deficiency ameliorates collagen-induced arthritis in mice. *J Immunol* 2002, 169:454-459
58. Johansson AC, Sundler M, Kjellen P, Johannesson M, Cook A, Lindqvist AK, Nakken B, Bolstad AI, Jonsson R, Alarcon-Riquelme M, Holmdahl R: Genetic control of collagen-induced arthritis in a cross with NOD and C57BL/10 mice is dependent on gene regions encoding complement factor 5 and FcgammaRIIB and is not associated with loci controlling diabetes. *Eur J Immunol* 2001, 31:1847-1856
59. Gebbia JA, Monco JC, Degen JL, Bugge TH, Benach JL: The plasminogen activation system enhances brain and heart invasion in murine relapsing fever borreliosis. *J Clin Invest* 1999, 103:81-87
60. Ploplis VA, Castellino FJ: Nonfibrinolytic functions of plasminogen. *Methods* 2000, 21:103-110
61. Ploplis VA, French EL, Carmeliet P, Collen D, Plow EF: Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. *Blood* 1998, 91:2005-2009