The Urokinase Plasminogen Activation System in Rheumatoid Arthritis: Pathophysiological Roles and Prospective Therapeutic Targets

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Abstract: Rheumatoid Arthritis (RA) is a chronic and progressive inflammatory disease characterized in its early stages by synovial hyperplasia and inflammatory cell infiltration and later by irreversible joint tissue destruction. The Plasminogen Activation System (PAS) is associated with a wide range of physiological and pathophysiological states involving fibrinolysis, inflammation and tissue remodeling. Various components of the PAS are implicated in the pathophysiology of RA. Urokinase Plasminogen Activator (uPA) in particular is a pro-inflammatory mediator that appears to play an important role in the bone and cartilage destruction associated with RA. Clinical studies have shown that uPA and its receptor uPAR are overexpressed in synovia of patients with rheumatoid arthritis. Further, genetic knockdown and antibody-mediated neutralization of uPA have been shown to be protective against induction or progression of arthritis in animal models. The pro-arthritic role of uPA is differentiated from its haemodynamic counterpart, tissue plasminogen activator (tPA), which appears to play a protective role in RA animal models. This review summarises available evidence supporting the PAS as a critical determinant of RA pathogenesis and highlights opportunities for the development of novel uPAS-targeting therapeutics.

Keywords: Rheumatoid arthritis, urokinase plasminogen activator, uPA, uPAS, plasminogen activation, drug targets.

1. INTRODUCTION

Rheumatoid Arthritis (RA), a common inflammatory disease with a worldwide incidence of ~0.3-1%, is characterized by chronic symmetrical polyarthritis, which progressively leads to irreversible joint destruction through synovitis-mediated cartilage degradation and bone erosion [1-3]. Significant advances in RA treatment have been made over the last two decades with the introduction of targeted biologic therapies, such as the anti-TNFα antibody Humira, and improved treatment regimens [2, 3]. Despite this progress, a significant percentage of patients are not adequately treated by current disease-modifying anti-rheumatic drugs (DMARDs). Identification and validation of new therapeutic targets and drugs is therefore required to improve RA therapy in the future [1]. Experimental models and clinical studies suggest that the Plasminogen Activation System (PAS) plays a pivotal role in RA disease progression. This review begins with an overview of the PAS before summarising available clinical data implicating this system in RA disease severity and progression. The reported effects of PAS genetic and pharmacological manipulation in various experimental models are then detailed, and a commentary on the relevance of the models in terms of recapitulating human RA is provided. Finally, the conclusion that the uPAS (urokinase plasminogen activation system) plays a key role in RA progression is critically discussed in the context of developing uPAS-targeting therapeutics for RA.

2. THE PLASMINOGEN ACTIVATION SYSTEM (PAS) AND CLINICAL EVIDENCE FOR ITS ROLE IN RA

2.1. Overview of the PAS

Activation of the zymogen plasminogen to the broad-spectrum serine protease plasmin can occur through two specific activators, tissue-type and urokinase-type plasminogen activator (tPA and uPA, respectively) [4-6]. tPA is primarily involved in intravascular fibrin clot dissolution. The fibrin clot acts as a template for binding and co-localization of both plasminogen (Plg) and tPA, which greatly stimulates Plg activation (PA). The resulting plasmin is protected from its circulating inhibitors (e.g. α2-antiplasmin) until the clot has been completely digested. In contrast, uPA is primarily involved in tissue remodeling and inflammation in a variety of physiologic states (e.g. wound healing, endometrial shedding), where it controls activation and inhibition of the pathway. Dysregulated expression and inhibition is linked to multiple pathologic states (e.g. invasive cancer, inflammatory disorders) [6-9].

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After binding to its cognate cell surface receptor uPAR, the pro-uPA single chain zymogen is converted via proteolysis into active two-chain uPA, which controls the activation of cell surface co-localised Plg to plasmin (Fig. 1) [4, 10]. While bound to the cell surface, uPA is protected from inhibition and activates plasmin, which subsequently triggers the activation of multiple downstream extracellular proteases (e.g. matrix metalloproteinases, collagenases), latent growth factors and other receptors (e.g. PARs). This results in directional remodeling of the local extracellular environment and signaling pathways (e.g. MAPK and/or JNK/STAT) driving cell proliferation, adhesion and migration [4-6, 9, 11-14]. These signaling pathways and downstream cellular events can also be modulated via complex direct and indirect interactions of uPAR with vitronectin in the ECM and a range of cell surface co-receptors, including integrins and growth factor receptors [5, 15, 16]. Growth factors, hormones, and inflammatory mediators including cytokines can, in turn, influence the expression of PAS genes [6, 13, 17-22], which can also be modulated via complex direct and indirect interactions of uPAR with vitronectin in the ECM and a range of cell surface co-receptors, including integrins and growth factor receptors [5, 15, 16]. Growth factors, hormones, and inflammatory mediators including cytokines can, in turn, influence the expression of PAS genes [6, 13, 17-22], which is thought to drive malignant tumor progression [23-25]. As receptor bound plasmin is protected from inhibition by α2-antiplasmin, efficient inhibition of uPA (and tPA) by two serine proteinase inhibitor (serpin) family members, plasminogen activator inhibitor-1 (PAI-1/SerpinE1) and -2 (PAI-2/SerpinB2) act as key regulators of pericellular PA [8]. Both form a covalent complex with uPA/uPAR causing internalisation of the entire complex via endocytosis receptors. Unlike PAI-2, inhibition of uPA by PAI-1 induces secondary high-affinity interactions with endocytosis receptor family members, with subsequent activating effects on cell migration and proliferation [26, 27]. This and other secondary binding mechanisms are possible explanations for why over-expression of PAI-1 is correlated with poor tumour prognosis [8, 28].

2.2. Clinical Findings

Evidence accumulated over the past three decades implicates the PAS in the clinical progression of RA, with many studies showing that expression of uPA, uPAR and PAI-1 is strongly upregulated in synovial tissue/knee aspirates of RA patients (Table 1). Relative to healthy controls, protein levels for these uPAS components in synovial fluid are increased 3-4-fold, with similar findings in knee cartilage extracts [17, 29-33]. Upregulation of uPA in the Synovial Fluid (SF) correlates with increased levels of active MMP-13 (collagenase-3), a plasmin substrate that is also implicated in RA pathogenesis [30]. The highly localized expression of uPA within diseased joints is evident from studies comparing knee aspirates with blood samples from the same patients, where SF uPA is increased as much as 4-fold over that found in circulation [17, 31]. Upregulation of uPA associates with disease severity, with uPA levels being the highest in the serum and SF of patients with radiographically-confirmed erosive disease, and correlates with Rheumatoid Factor (RF) expression in these patients [31]. uPA and uPAR levels are increased in the synovial fluid of RA patients relative to osteoarthritis (OA) patients and healthy controls [29, 34-36]. Furthermore, high levels of uPA activity were detected in knee cartilage extracts taken from terminal RA patients who had received total knee arthroplasties [32]. Patient-derived synovial fibroblasts produce large amounts of uPA and uPAR in vitro [37] and uPA proteolytic activity localizes to the hyperproliferative synovial lining in patient joint sections [35]. Similarly, serum soluble uPAR (suPAR; released by cleavage of uPAR by uPA or plasmin or shed in intact form cells [38]) is increased in RA relative to patients with other inflammatory rheumatic diseases (e.g. Sjögren’s syndrome) and healthy controls and has prognostic significance as a biomarker of erosive progression [42, 43]. Similarly, PAI-2 levels correlate with increased Larsen score severity, increased cytidine deaminase activity and leukocyte counts in SF samples from RA patients [33, 40]. In addition, serum suPAR has also shown utility as a treatment-response biomarker for monitoring adalimumab therapy, with responders showing significantly decreased suPAR levels after 8 weeks of treatment [47].

In contrast to uPA, expression levels and/or activity of tPA are generally decreased in RA SF relative to healthy or OA synovium [39]. Similarly, tPA proteolytic activity was undetectable in articular cartilage derived from RA patients who had undergone total knee arthroplasty [32]. Only one study found significantly increased tPA antigen levels in RA relative to OA samples, where uPA, uPAR and PAI-1 antigen levels were also significantly increased [29]. This down-regulation of tPA expression may be localized to arthritic joints as circulating tPA, along with PAI-1, are significantly increased in the blood of RA patients and correlate with a greater risk of hypertriglyceridaemia and insulin resistance.

![Fig. (1). Schematic overview of the urokinase plasminogen activation system (uPAS). ECM = extracellular matrix, MMP = matrix metalloproteinase, PAI = plasminogen activator inhibitor, uPA = urokinase plasminogen activator, uPAR = urokinase plasminogen activator receptor. (The color version of the figure is available in the electronic copy of the article).](image-url)
Table 1. Summary of PAS and other component expression in RA patient tissue samples.

| PAS and Other Components | Technique | Finding | Refs. |
|--------------------------|-----------|---------|-------|
| tPA, uPA                 | ELISA     | In RA SF tPA ↓ 4-fold, PAI-1 activity ↓ 4-fold, total uPA antigen ↑ >4-fold and pro-uPA ↑ 3-fold relative to plasma levels in the same patients or healthy controls. Active uPA was detected in 14 of 36 RA SF samples. | [17] |
| uPA, tPA, PAI-1, PAI-2   | ELISA, radiography | uPA, PAI-2 and PAI-1 ↑ in RA SF. uPA increased in RA but not OA. tPA level low in RA and OA. No correlation between uPA or PAI levels and SF proteoglycans (products of cartilage degradation). uPA activity correlated with ↑ CD activity. | [33] |
| uPA, PAI-1, TP, WBC      | ELISA     | WBC ↑ 44-fold, TP ↑ 1.3-fold, uPA ↑ 2.5-fold and PAI-1 ↑ 4.6-fold in RA SF compared to OA SF. uPA levels strongly correlated with PAI-1 level in RA SF. | [36] |
| uPA, uPAR, PAI-1, PAI-2, tPA | ELISA, SDS-PAGE | uPA, uPAR, PAI-1 and tPA ↑ in RA knee aspirates compared to OA and healthy controls. uPAS expression in RA patients similar to those with pseudogout. | [29] |
| uPA, PAI-1, MMP-1, MMP-3, TIMP-1, TIMP-2 | ELISA, Northern Blot | uPA ↑ 3-fold, PAI-1 ↑ 3.5-fold in knee cartilage extracts from RA patients. uPA, PAI-1, MMP-1, MMP-3, TIMP-1, TIMP-2 mRNA detected in the articular cartilage of all RA patients. | [32] |
| uPA, uPAR, tPA, PAI-1, PAI-2 | ELISA, IHC | uPA ↑ 230-fold, uPAR ↑ 2-fold, PAI-1 ↑ 4.7-fold and tPA ↓ 4.5-fold in RA SF compared to OA SF. PAI-2 detected in RA SF, undetectable in OA. RA synovial tissue stained strongly for uPA, uPAR and PAI-1. Moderate staining for tPA in OA and RA synovial tissue – localized to endothelia. | [39] |
| uPA, uPAR, tPA, PAI-1, PAI-2, Plg, Fib-D-dimer | ELISA, BIA, histology and Radiography | Pro-uPA ↑ 3-fold, Plg ↑ 3-fold, PAI-1 ↑ 9-fold, Fib D-dimer ↑ 54-fold, PAI-2 detected in SF. tPA undetectable in SF, 0.74 IU/mL in plasma. Plasma PAI-2 correlated with increased severity (Larsen score). | [40] |
| uPA, uPAR, tPA, PAI-1 | Gel and in situ zymography, IHC, Northern blot and ISH | tPA mediated proteolytic activity predominant in healthy controls and OA, but reduced in RA patients. uPA mRNA, antigen and activity predominant in RA proliferative synovium. uPAR and PAI-1 expression ↑ in RA synovium. | [35] |
| uPAR                     | IHC       | RA synovial lining, endothelium and interstitial macrophages uPAR+. Healthy controls, low numbers of myeloid cells uPAR+. | [41] |
| suPAR                    | ELISA     | Serum suPAR ↑ in RA serum relative to health controls or ReA and PSS patients. suPAR positively correlated with CRP, ESR and the number of swollen joints in RA. | [42] |
| suPAR                    | ELISA     | Serum suPAR ↑ in RA patients with erosive pathology compared to patients without erosive progression. suPAR suggested as a serum biomarker of erosive progression in RA. | [43] |
| uPA, RF                  | ELISA     | Serum and SF uPA ↑ relative to healthy controls. SF uPA ↑ 2-fold compared to matched serum levels in non-erosive and erosive RA. Serum and SF uPA highest in erosive RA. uPA positively correlated with RF status in erosive RA. | [31] |
| SNPs in PLAU             | PCR, nephelometry, radiography. | ↑ C/T SNP at nucleotide +4065 in PLAU 3'-UTR relative to healthy controls. No correlation between C/T SNP and RF positivity, extra-articular involvement or bone erosion found. | [44] |
| uPA, uPAR, PAI-1         | ELISA, gel zymography | uPA, uPAR and PAI-1 ↑ in knee aspirates of patients with gouty arthritis, correlation with ↑ MMP-9 activity. | [45] |
| uPA                     | IHC, ELISA, Radiography | uPA expression correlates with survivin expression in blood and SF from patients with erosive RA. | [46] |
| uPAR                    | ELISA     | ↓ serum uPAR correlated to treatment responsiveness in RA patients receiving anti-TNFα antibody Adalimumab after 8 weeks. No difference in serum uPAR level for non-responders to Adalimumab. Baseline serum uPAR highest in non-responders. | [47] |
| uPA, MMPs 1, 2, 3, 7, 9, 13 | ELISA | uPA ↑ 3-fold in RA SF relative to OA. uPA antigen levels positively correlated with ↑ MMP-13 in RA SF. | [30] |

CD = cytidine deaminase, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, Fib D-dimer = Fibrin D-dimer, IHC = immunohistochemistry, ISH = in-situ hybridisation, MMP-1 = matrix metalloproteinase 1, MMP-2 = matrix metalloproteinase 2, MMP-3 = matrix metalloproteinase 3, MMP-13 = matrix metalloproteinase 13, OA = osteoarthritis, PAI-1 plasminogen activator inhibitor 1, PAI-2 plasminogen activator inhibitor 2, PSS = primary Sjögren’s syndrome, RA = rheumatoid arthritis, RF = rheumatoid factor, ReA = reactive arthritis, sc-uPA = single-chain uPA, SF = synovial fluid, SNP = single nucleotide polymorphism, suPAR = soluble urokinase plasminogen activator receptor, uPA = urokinase plasminogen activator, uPAR = urokinase plasminogen activator receptor, 3’UTR = 3’-untranslated region, TIMP1 = TIMP metalloproteinase inhibitor 1, TIMP2 = TIMP metalloproteinase inhibitor 2, TNFα = Tumour necrosis factor alpha, TP = total protein, tPA = tissue plasminogen activator, WBC = white blood cell count.
This pattern contrasts to that seen in OA, where tPA activity is strongly dominant over uPA, or in healthy synovium where uPA activity is undetectable [35]. In RA synovium, high levels of uPA activity are observed throughout the entire synovial membrane, whereas in OA tPA activity is confined to the luminal face of the vascular endothelium [35]. As such, the predominance of uPA over tPA in the affected joints appears to differentiate the reactive arthritides, like RA and Sjögren’s syndrome, from OA, where tPA expression dominates [42]. As the primary physiological role of tPA is to facilitate fibrin clearance via direct binding to fibrin clots [49], its downregulation in RA has implications for fibrin turnover [50]. Inappropriate fibrin deposition is a diagnostic characteristic in RA joints [51] (referred to as rice bodies) and is believed to promote sustained inflammation and tissue damage in the synovial space (reviewed in [52]). Indeed, the balance between uPA/tPA expression appears to play a key role in the dysregulation of fibrin clearance in diseased synovium and the wider extravascular coagulation observed in the joints of RA sufferers.

3. EFFECTS OF PAS IN RA MOUSE MODELS

Rodent models of RA developed over the past five decades have provided significant insights into arthritis pathophysiology. Inducible models of RA span from acute monoarticular models, where a single joint is affected, to systemic models with multiple joint involvement, similar to the pathology most commonly observed in human RA patients [53]. In addition, several spontaneous models of arthritis in genetically modified mice have been reported [54]. Studies of the involvement of the PA system in RA pathology have for the most part focussed on the use of two common models; Collagen-Induced Arthritis (CIA) and Antigen-Induced Arthritis (AIA). CIA, a systemic polyartritic model featuring symmetrical involvement of both proximal (knee) and distal (phalangeal) joints, is one of the most commonly used experimental models of RA [55, 56]. CIA is initiated by intradermal injection of heterologous collagen-II in complete Freund’s adjuvant (CFA) into the tail of susceptible mouse strains (e.g. DBA1 or C57BL/6), which is heavily dependent on MHC-II haplotype [57, 58], followed by an intraperitoneal booster injection up to 21 days later. As an autoimmune model, CIA is primarily driven by activation of collagen-specific B cells that produce cross-reactive anti-CII-antibodies directed to endogenous antigen in the mice [55]. Binding of collagen in the joint by these antibodies triggers a local inflammatory response via immune complex formation [59] and complement activation [60], promoting infiltration of circulating immune cells, chronic inflammation and tissue damage [61]. AIA is similar to CIA in that pathology relies on the sensitization of an adaptive immune response, although the antigen, in this case, is not an endogenously expressed self-protein. In AIA, methylated Bovine Serum Albumin (mBSA) in CFA is administered (intradermal) into the base of tail on days 0 and 7, along with intraperitoneal heat-killed Bordetella pertussis as an additional adjuvant [62]. Intra-articular injection of mBSA in saline into the knee initiates local pathology in the affected joint, while injection of saline into the contralateral knee provides a matched intra-individual control. The positively charged mBSA binds to negatively charged cartilage in the joint, directing a T-cell-dependent adaptive immune response towards the joint resulting in local inflammation [63, 64]. Both CIA and AIA recapitulate a variety of the clinical and histological features of human RA, including synovitis, synovial hyperplasia and pannus formation, immune cell infiltration and progressive cartilage and bone erosion [62, 65-68]. However, the requirement for traumatic delivery of antigen into the joint and its monoarticular nature set AIA apart from both CIA and the clinical scenario in the majority of RA patients [65]. In addition, and in contrast to RA and CIA, AIA is a transient model, where inflammation ceases after mBSA in the joint is depleted, although pathology may be prolonged by repeat injection [63, 69]. A summary of studies concerned with the differing functions of the PAS and the consequences of manipulating various components in mouse models of RA, is presented in Table 2.

3.1. AIA and Septic Arthritis

The contrasting roles of PA in acute versus systemic models of RA have been the subject of debate for over two decades [12]. Multiple groups have shown that in acute antigen-induced models of monoarticular RA, uPA appears to play a protective role [70], as uPA-/- mice show significantly worse pathology [71]. Here, uPA is believed to play a primary role in fibrinolysis within the affected joints as fibrin deposition is significantly increased in uPA-/- mice, correlating with increased disease severity [71, 72]. Furthermore, PAI-1-/- mice show significantly milder symptoms and decreased fibrin deposition in response to AIA [73], supporting that uPA activity is protective in these models via increased fibrinolysis. Similarly, tPA deficiency in the acute mBSA/IL-1 monoarticular model [71] and Plg deficiency in the classic AIA model [70] both resulted in worsened disease, characterized by increased fibrin deposition in joints. Thus, it appears that plasmin-mediated fibrinolysis is an essential protective mechanism in models involving intrarticular administration of mBSA. Similar results have been reported in a monoarticular model of septic arthritis, where Plg-/− mice showed reduced clearance of bacteria and necrotic tissue from affected joints. However, in contrast to AIA, differences in fibrin deposition between Plg-/- and WT mice were not seen, suggesting that the protective effects of Plg are not a direct result of its fibrinolytic action in this model [74]. In a separate study, inhibition of Plg cell surface binding using tranexamic acid was found to exacerbate arthritic symptoms after systemic administration of Staphylococcus aureus [82].

3.2. CIA and Systemic Models of Polyarthritis

A distinctly different pattern is seen for the components of PAS in non-septic systemic models of RA, including CIA, CAIA and KBxN serum transfer-induced arthritis. Aside from their similarities in modeling the systemic polyarthritis observed in human patients, these models all require immune complex formation and consequent C5 activation for disease initiation [85, 86]. In this sense, they differ from AIA, where a humoral immune response is not required for the manifestation of pathology [87], despite the observation of immune complex formation in this model [88].

In CIA, Plg itself is essential for the initiation of symptoms, with Plg −/− DBA1 mice showing no signs of arthritic
Table 2. Summary of PAS involvement in mouse models of RA.

| Arthritis Model | Model/Background | Modification/Intervention/PAS Components | Parameters Measured | Outcomes | Refs. |
|-----------------|------------------|------------------------------------------|---------------------|----------|-------|
| CIA/CAIA/KBxN serum transfer (systemic polyarthritis) | CIA/ uPA-/- or Plg-/- mice on C57BL/6 × DBA/1J (backcrossed 1 and 2×, respectively) | uPA-/- Plg-/- | ELISA for anti-CII antibodies, histology, clinical score. | ↓ Incidence and severity: uPA-/- > uPA+/- > WT. Supplementation of Plg-/- with exogenous Plg increased susceptibility to CAIA | [75] |
|                 | CIA, CAIA and KBxN/uPA-/- mice on C57BL/6 | uPA-/- Bone marrow chimeras | Histology, clinical score, inflammatory cytokines. | uPA from a bone marrow-derived cell lineage required for CIA (WT→uPA-/-). uPA-/- mice resistant to CAIA and KBxN serum transfer-induced arthritis. | [76] |
|                 | CIA/ uPA-/- or tPA-/- mice on C57BL/6 | uPA-/- tPA-/- Bone marrow chimeras | Histology, clinical score, anti-CII ELISA, T-cell proliferation, joint inflammatory cytokines. | ↓ Severity in uPA-/-, ↑ Severity in tPA-/-, ↑ histology scores in WT, histology scores higher in tPA-/- > WT. ↓ IFN-γ levels in CII-specific T-cell culture supernatants from uPA-/- | [72] |
|                 | CIA/ uPA-/-, uPAR-/- mice on DBA/1J (backcrossed 8 ×) | uPA-/- uPAR-/- Bone marrow chimeras | Histology, clinical score, inflammatory cytokines. | Near complete amelioration of joint disease in uPA-/- and uPAR-/- (uPA-/-→uPAR-/-) vs WT mice. Bone marrow chimeras demonstrated uPAR expression by a bone marrow-derived cell lineage was sufficient for CIA (WT→uPAR-/-), whereas uPAR from all other sources did not cause arthritis (uPAR-/-→WT). | [77] |
|                 | CIA/DBA/1 mice | Anti-uPA or anti-uPAR mAb | Histology, clinical score, inflammatory cytokines. | Anti-uPA MAb reduced symptoms to same extent as Etanercept in CIA. Anti-uPAR MAb had no effect. | [78] |
|                 | CIA/ DBA/1 mice | uPA mATF-HSA fusion protein | Histology, radiology, clinical score. | ↓ CIA incidence and clinical score in treated mice. | [79] |
|                 | CIA/ DBA/1J mice | Nil | VEGF PAR-1, TFPI, EGR1 and uPA mRNA. | ↑ uPA and PAI-1 expression in CIA mice. | [80] |
| Delayed-type Hypersensitivity DTH (Acute single paw) | female C57BL/6 mice | Anti-uPA or Anti-uPAR mAb | Histology, clinical score, inflammatory cytokines. | Anti-uPA mAb significantly decreased histological synovitis, bone erosion and cartilage destruction but did not decrease clinical score. Anti-uPAR mAb had no effect. | [78] |
|                 | AIA (Acute monoarticular) | uPA-/- or Plg-/- mice on C57BL/6J (backcrossed 6 ×) | uPA-/- Plg-/- Histology, in situ zymography, 99Tc uptake | ↑ uPA activity in arthritic synovial membrane. ↑ 99Tc uptake and histological scores in Plg-/- and uPA-/- mice. ↓ Bone erosion, cartilage destruction and synovial thickness in Plg-/- and uPA-/- | [70] |
|                 | C57Bl/6 mice | uPA | VEGF, PAR-1, TFPI, EGR1 and uPA mRNA | Systemic hypercoagulable state, more severe in AIA than in CIA. uPA expression ↑ in early stages with decrease to baseline over time. | [80] |
|                 | PAI-1-/- mice | PAI-1-/- | Histology, tissue fibrin D-dimer quantitation, in situ zymography, 99Tc uptake | ↓ Histological score, ↓ 99Tc uptake, ↓ cartilage destruction, ↓ fibrin deposition in joints in PAI-1-/- mice. ↓ D-dimer and ↑ tPA activity in PAI-1-/- mice. | [73] |
|                 | uPA-/- or tPA-/- mice on C57BL/6 | tPA-/- uPA-/- | Histology, clinical score, inflammatory cytokines | ↑ arthritis scores in tPA-/- and uPA-/- mice, most severe in tPA-/-, ↑ fibrin deposition in uPA-/- and tPA-/- mice. | [71] |

(Table 2) contd....
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| Arthritis Model                                      | Model/Background          | Modification/Intervention/Components | Parameters Measured                                      | Outcomes                                                                 | Refs. |
|------------------------------------------------------|---------------------------|-------------------------------------|----------------------------------------------------------|--------------------------------------------------------------------------|-------|
| Plg-/- mice on C57BL/6 × DBA/1J mice (backcrossed 2 ×) | Plg-/-                    | Histology, clinical score, inflammatory cytokines. | ↑ clinical scores, bone erosion, cellular infiltration, proteoglycan loss and histological scores in uPA-/- mice. ↑ and sustained expression of IL-1β, TNFα, MMP3 and MMP13 (uPA-/- > C57BL/6) ADAMTS-4 gene expression in uPA-/- < C57BL/6 ↑ DIPEN staining uPA-/- in AIA (uPA-/- > C57BL/6) | [66]  |
| uPA-/- mice on C57BL/6                               | uPA-/-                    | Histology, clinical score, inflammatory cytokines. | ↓ clinical and histological scores in uPA-/- mice. | [81]  |
| S. aureus cell wall induced arthritis ( intra-articular ) Plg-/- mice | Plg-/-                    | Histology, clinical score, inflammatory cytokines. | ↑ inflammation, tissue destruction, and bacterial growth in Plg-/- mice. Administration of exogenous human Plg enhanced bacterial clearance and ↓ necrotic tissue accumulation in joints. | [74]  |
| Streptococcal septic arthritis ( systemic )/NMRI mice | Tranexamic acid            | Histology, plasmin activity, survival. | Tranexamic acid ↑ arthritis severity, ↓ survival. Administration of exogenous tPA or plasmin did not rescue mice from staphylococcal sepsis. ↑ D-dimer levels under staphylococcal sepsis. | [82]  |
| LIA using CII/Plg-/- mice on C57BL/6 × DBA/1J (backcrossed 2 ×) | Plg-/-                    | Histology, clinical score, inflammatory cytokines. | Moderate arthritic symptoms in WT, no arthritis in Plg-/- mice. Intra-articular injection of saline or CII resulted in mild arthritic symptoms in the injected joint in Plg-/- and exacerbated disease in WT. CII more severe than saline in Plg-/- and WT. Severity correlated with increased Fib deposition in joint. | [66]  |
| KBxN serum transfer + intra-articular saline injection/C57BL/6 and uPA-/- mice on C57BL/6 | uPA-/-                    | Histology, clinical score, inflammatory cytokines. | ↓ clinical and histological scores in uPA-/- mice. ↓ proteoglycan loss and fibrin deposition in uPA-/- mice receiving intra-articular saline. | [81]  |
| Patient-derived RA synovial fibroblasts and cartilage engrafted into SCID mice | uPA inhibitor WX-340 uPAR As-ODN | Cartilage invasion in vivo, histology. | ↓ cartilage invasion with uPAR As-ODN. uPA-specific small molecule inhibitor had no effect. | [83]  |
| Tg197 TNF α human transgenic mice Plg-/-, Fib-/-, Plg/-, Fib-/- mice (backcrossed at least 7×) | Plg-/- Fib-/- Plg/-, Fib/- | Histologic score, arthritic score. | ↑ incidence and severity of arthritis in paw joints but ↓ severity in knee joints of Plg-/- Tg197 mice. ↓ MMP-9 activity in knees but not paws of Plg-/- Tg197 mice. Superposition of Fib-/- onto Plg-/- background simultaneously reversed the pro-arthritic phenotype in paws and resistant phenotype in knees of Plg-/- Tg197 mice. | [84]  |

ADAMTS4 = a disintegrin and metalloproteinase with thrombospondin motifs 4, AIA = Antigen-induced arthritis, As-ODN = antisense oligonucleotide, CAIA = collagen antibody induced arthritis, CIA = collagen induced arthritis, CII = collagen type II, DIPEN = metalloproteinase induced neoeptitope Asp-Ile-Pro-Glu-Asn341', DTH = delayed type hypersensitivity, EGR1 = early growth response 1, Fib = fibrinogen, INF-γ = interferon γ, LIA = localized injury induced arthritis, mATF-HSA = murine uPA amino-terminal fragment-human serum albumin fusion protein, MMP = matrix metalloproteinase, PAI = plasminogen activator inhibitor, PAR-1 = protease activated receptor-1, PDX = patient-derived xenograft, Plg = plasminogen, tPA = tissue-type plasminogen activator, TFPI = tissue factor pathway inhibitor, TNFα = tumour necrosis factor α, uPA = urokinase-type plasminogen activator, uPAR = urokinase-type plasminogen activator receptor, VEGF = vascular endothelial growth factor, IFN = interferon.

disease after collagen immunization [66, 75]. Similarly, Plg-/- DBA1 mice are insensitive to the administration of CII auto-antibodies (i.e. CAIA), with full disease severity able to be restored by daily administration of exogenous human Plg, suggesting a role for Plg in the effector phase of inflammation [75]. While not completely devoid of CIA symptoms, uPA-/- mice typically show only mild symptoms in the CIA model, along with significantly decreased disease incidence.
compared to WT [66, 72, 75]. Reduction of symptoms is gene dosage-dependent (i.e. \( uPA^{-/-} < uPA^{+/+} < WT \)) and is affected by the number of backcross generations onto a susceptible genetic background (\( ↓ \) generations = milder symptoms). \( uPA \)-deficient mice backcrossed onto C57BL6 mice 8 times showed minimal CIA symptoms (mean clinical score at endpoint \( = 1.5 \pm 0.6 \), WT \( = 4.1 \pm 1.2 \)) and complete resistance to arthritis mediated by serum transfer from KBxN mice or CAIA [76]. Bone marrow chimera experiments revealed that \( uPA \) produced by a bone marrow-derived cell lineage was required to elicit disease symptoms (C56BL6→\( uPA^{-/-} \)) in the CIA model, while the reciprocal engraftment (\( uPA^{-/-} \rightarrow C56BL6/6 \)) showed that \( uPA \) from all other host sources is not sufficient to cause disease [76]. From this, it was proposed that monocytes/macrophages were the most likely source of pro-arthritic \( uPA \), given that lymphocytes are not required for disease induction in either the CAIA or KBxN models [89].

The essential nature of \( uPAS \) function in CIA induction was further demonstrated in a recent report using \( uPA^{-/-} \)-mice backcrossed onto the DBA1 background, which showed virtually no clinical arthritic symptoms and very mild histological scores following CII immunization [77]. Large decreases in incidence, symptom severity and microscopic disease in knees were also observed for \( uPAR^{-/-} \)-crosses, although to a lesser extent than in \( uPA^{-/-} \)-mice. Consistent with a diminished inflammatory response, significant decreases in paw mRNA levels for key inflammatory cytokines were observed for both genotypes, along with minimal fibrin deposition in joints. Humoral and T-cell responses were found to be unaffected in both genotypes, with the exception of a modest decrease in IgG2a titres in \( uPA^{-/-} \)-mice following CII challenge. Similar to observations in \( uP A^{-/-} \)-C57BL mice [76], bone marrow chimera experiments revealed a crucial role for bone marrow derived \( uPAR \) expression in CIA pathology [77]. WT animals reconstituted with bone marrow transplants from \( uPAR^{-/-} \)-mice (\( uPAR^{-/-} \rightarrow \) DBA1) showed large decreases in arthritis incidence and severity relative to WT chimeras (DBA1→DBA1). Reciprocal chimeras (DBA1→\( uPAR^{-/-} \)) demonstrated that \( uPAR \) competency within bone marrow derived cells is needed to elicit disease, although the resulting arthritis symptoms showed lower incidence and were milder than in control mice. Monocytes/macrophages were again suggested as the myeloid cell lineage most likely to be responsible for the requisite expression of pro-pathogenic \( uPAR \).

Aside from the known pro-inflammatory role of macrophages in rheumatoid arthritis [90], support for this hypothesis comes from observations that macrophage \( uPA \) and \( uPAR \) expression is induced by inflammatory cytokine exposure [19, 20, 91, 92]. Macrophages accumulate in diseased synovium [93] where they mediate joint specific inflammation via local expression of pro-inflammatory cytokines (like TNFn) [90, 94] and proteases linked to clinical RA progression (e.g. MMP-9) [95]. Indeed, selective macrophage depletion has been investigated as a strategy for the clinical treatment of RA [96, 97]. Support for the strategy comes from studies in the CIA mouse model showing that systemic targeting of macrophages [98] or synovial macrophage-like cells [99] ameliorates disease progression. Similarly, macrophage cellularity in arthritic joints is known to decrease in response to clinical pharmacotherapy [100].

In contrast, \( tPA^{-/-} \) mice show considerably worse pathology in CIA, characterized by increased clinical scores, joint fibrin deposition and IL-1β expression [72]. Thus, the divergent effects of \( tPA \) and \( uPA \) knockouts suggest that \( tPA \) is the primary mediator of fibrinolysis in both systemic (CIA) and acute (AIA) models of arthritis. In comparison, through its co-expression with \( uPAR \) by infiltrating myeloid cells, \( uPA \) appears to play a decisively pro-arthritic role in multiple systemic immune complex-mediated models of RA, primarily mediated through downstream Plg activation.

### 3.3. Hybrid Models

Insight into the differential roles of the PAS components in acute versus systemic models has been provided by hybridized approaches that incorporate intra-articular injury and/or antigen administration into systemic models of RA. Li and co-workers reported a novel modification to the common AIA protocol, where mBSA was replaced by CII for initial immunization and as the triggering antigen [66]. In this model (dubbed local injection-induced arthritis, LIA) WT mice developed mild arthritic symptoms, whereas direct intra-articular injection of either saline or CII resulted in more severe pathology [66]. In line with observations from conventional CIA models, Plg-/- mice were found to be resistant to arthritis in the absence of local injury [66, 75]. However, intra-articular injection of saline was found to elicit mild arthritic symptoms and fibrin deposition in affected joints. Injection of CII into the joint resulted in more severe symptoms, although still less than that seen for either intervention in WT C57BL. These results demonstrate that the trauma related intra-articular injection was alone sufficient to cause arthritic pathology and fibrin deposition, even in the absence of functional Plg.

Modification of the KBxN serum-transfer model to include a local monoarticular injury has provided further evidence for the pathogenic role of \( uPA \) in systemic models of RA that depend upon immune complex formation [101] and complement activation [86] (recently reviewed in [102]). While normally resistant to arthritis mediated by KBxN serum transfer, arthritic pathology was able to be triggered by the intra-articular injection of saline into the knees of \( uPA^{-/-} \)-C57BL6 mice [81]. As saline injection itself is not thought to be arthritogenic based on findings in AIA [103] or mBSA/IL1 [104] monoarticular models, injection associated injury and resulting fibrin deposition appears to act as a catalyst for symptom onset in the absence of \( uPA \). In addition, the intra-articular injection of active Low Molecular Weight (LMW) \( uPA \) into the knees of healthy mice is able to elicit classic arthritis-like symptoms, including increased IL-1β, IL-6 and TNFα production, synovitis and pronounced pan-nus formation [31].

### 3.4. Tg197 TNFα-driven Model

Recently, Plg was found to act as a regiospecific modulator of arthritis progression in the TNFα-driven Tg197 humanized mouse model, a well-studied spontaneous model of RA [105]. Both disease incidence and severity were significantly exacerbated in the paws of Plg-/- Tg197 mice relative to Tg197 controls, which are prone to spontaneous arthritis [84]. The exact opposite effect was observed in the knee
joints of the same animals, however, where Plg-deficiency resulted in significantly decreased histology scores for all microscopic pathologies. Joint-specific differences did not correlate to differences in the expression of PAS components, with tPA and uPA mRNA levels similar across both paws and knees (tPA levels were slightly lower in knees relative to paws). The intriguing differential effects of Plg were instead correlated to increased fibrin deposition in both the paws and knees of Plg-/- Tg197 mice. The causative role of fibrin was elegantly demonstrated in experiments where the fibrinogen deficiency was superposed onto the Plg-/- Tg197 background. These showed that the deleterious effects of Plg-/- in paws and the protective effects of Plg-/- were able to be reversed through the removal of Fib, with histological indices at both sites being comparable to Plg+/+/Fib+/+ Tg197 controls. It was reasoned that fibrin may play a protective role in knee sites being comparable to Plg+/+/Fib+/+ Tg197 controls. It was reasoned that fibrin may play a protective role in knee pathology by supporting vascular integrity, minimizing the likelihood of local haemorrhage, and by providing a local matrix for reparative processes. These findings were in line with clinical observations of increased proximal joint arthropathies (e.g. knees, hips) in haemophilic patients [106-109]. Similarly, activation of MMP-9, a prominent pro-arthritic collagenase and plasmin substrate, was significantly inhibited in the knee joints of Plg-/- mice, whereas MMP-9 was not detected in the paws of the same animals. Together, these results suggest that the opposing consequences of Plg-/- in proximal versus distal joints of Tg197 mice may arise through multiple mechanisms.

4. TARGETING THE uPAS FOR RA THERAPY

The apparent pro-pathogenic role of the uPAS in KO mouse models of CIA has led to the suggestion that selective targeting of this system may be a viable approach for the development of new drugs for RA [12, 52, 72]. In particular, selective inhibition of uPA proteolytic activity has been put forward as a potentially efficacious strategy [12, 75, 76]. Early work in this area demonstrated that covalent inhibition of the uPA active site by the chloromethylketone PPACK significantly decreased arthritis symptoms caused by the intra-articular injection of LMW-uPA [31]. Again, the key role of circulating myeloid-derived immune cells in RA was demonstrated in this work as the depletion of monocytes with etoposide prior to and following injection of LMW-uPA resulted in an almost complete protection from arthritic symptoms. (Fig. 2).

Outside of targeting uPA proteolytic activity, the uPA/uPAR interaction has also been investigated in vivo. Virally induced expression of a fusion protein consisting of the uPAR-binding amino-terminal fragment of uPA and Human Serum Albumin (HSA) modestly inhibited CIA in DBA1 mice [79]. In addition, synovial fibroblasts derived from RA knee arthroscopy patients showed markedly decreased proliferation and invasiveness in vitro in response to uPAR-targeting antisense ODN or treatment with the active-site targeting small molecule uPA inhibitor WX-340 [83]. uPAR ODN inhibited RA SF invasion into human cartilage explants in the SCID RA xenograft model, whereas WX-340 did not decrease invasion.

A very recent report provided compelling evidence that uPA proteolytic activity is necessary for disease progression in the DBA1 CIA mouse model [78]. Using a murine mAb (mU1) capable of blocking enzymatic activation of pro-uPA and the catalytic activity of uPA, but not uPA binding to uPAR, the authors observed significantly reduced progression in DBA1 mice with existing arthritic symptoms [110]. Over a 14-day treatment, uPA inhibition reduced clinical and histopathologic scores to the same extent as the TNFα-targeting drug Etanercept. In addition, mU1 was trialled in a delayed hypersensitivity (DTH) DBA1 model [111], a tran-

**Clinical RA**

- Diseased joints
  - uPA, uPAR, PAI 1&2 †
  - tPA ‡

**Therapeutic Targets**

- Precedent: uPA Proteolytic Activity
  - Active-site targeting MaB = Etanercept in CIA

**RA Mouse Models**

- Monoarticular Models
  - Plg, uPA, tPA Anti-arthritic

**Systemic models**

- Plg, uPA, uPAR Pro-arthritic

**Infiltrating Myeloid cells**

- uPA and uPAR required for CIA/CAIA models

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**Fig. (2).** Clinical and experimental insights into the PAS in RA. Expression of uPAS components (uPA, uPAR, PAI-1 & 2) is upregulated in rheumatic joints, while tPA is decreased. KO mouse studies show conflicting evidence for the roles of PA and the uPAS from protective (AIA) to promoting (CIA, CAIA, KbxN serum transfer) RA symptoms. Arthritis severity in patients and mouse models relates to dysregulated fibrinolysis and increased fibrin deposition. Bone marrow chimera experiments with KO mice demonstrated that expression of uPA and uPAR by myeloid-derived cells is essential for susceptibility in CIA, reflecting the known pathogenic roles of macrophages and neutrophils in RA. Targeting of uPA proteolytic activity has shown efficacy equivalent to that of the approved anti-TNFα drug Etanercept. Together, this evidence supports uPAS components as novel drug targets for RA. (The color version of the figure is available in the electronic copy of the article).
sient local model developed from traditional AIA models. Here uPA, blockade significantly reduced histopathologic measures of synovitis, bone erosion and cartilage degradation, although the reductions did not result in significant decreases in clinical score. Etanercept in comparison was able to reduce macroscopic disease progression, significantly decreasing paw swelling relative to isotype controls. Interestingly, experiments in both the CIA and DTH models using an anti-uPAR mAb selective for the uPA binding site (mR1) showed no significant effects on disease progression [112]. This contrasts somewhat from previous work where the same mAb was efficacious in an anthrax toxin activation assay [112] and hepatic fibrinolysis assay in tPA−/− mice [113], experiments that both depend upon uPA proteolytic activity. Altogether, these studies support pharmacological targeting of uPA proteolytic activity as a promising approach to RA therapy.

CONCLUSION

Taken together, the clinical evidence coupled with insights from systemic (i.e. collagen-induced) arthritis models suggest a distinctly pro-arthritic role for the uPAS in RA (Fig. 1). Selective upregulation of uPAS components in affected joints appears to be a general characteristic of RA, and one that correlates with increased severity and disease progression. Furthermore, the observation that uPAS upregulation coincides with increased synovial fibrin deposition suggests that this system is ineffective in appropriately regulating local fibrinolysis, a task made all the more difficult by the concomitant downregulation of tPA. Thus, the clinical trends appear to resemble those from the systemic immune-complex mediated mouse models of RA, which appear most relevant to the polyarthritic symptoms observed in the clinic. In these models, expression of uPAS components is essential for the development of arthritis, whereas tPA appears to provide protection through regulation of local fibrinolysis. Further, the key role of myeloid-derived cells in RA pathogenesis is evident in these animal models and is supported by clinical studies using synovial sections in which uPA+ and uPAR+ subsets of macrophages and neutrophils were identified by colocalization with CD68 or MPO staining, respectively [78].

Despite the availability and development of several drugs over the last 3 decades for the treatment of RA, including anti-TNFα biologics, IL-6 inhibitors, methotrexate and leflunomide, which can reduce RA progression and joint destruction, the unfavorable therapeutic profiles and low rates of disease remission have remained a major challenge for clinicians [114-118]. Methotrexate alone or in combination with other DMARDs continues as the mainstay treatment for a large population of RA patients, but liver and renal toxicities are a major concern [118]. Similarly, while TNFα-targeting therapies have helped revolutionize the therapeutic landscape for RA, durable responses are not observed in a large proportion of patients [119]. In addition, increased risks of serious infection and malignancies limit the appeal of anti-TNFα drugs [120]. Thus, there remains a considerable unmet need for the development of new drugs with better efficacy and safety profiles. As the proteolytic activity of uPA appears to be important in the development of CIA, and inhibition of uPA activity can ameliorate arthritis symptoms in animal models, highly selective and non-toxic uPA inhibitors that lack the immunosuppressive side effects and the dosing issues characteristic of biological DMARDs represent attractive candidates. We recently reported highly selective and drug-like small molecule uPA inhibitors that inhibit uPA-driven metastasis in vivo [121]. Studies of the efficacy of these inhibitors in a rat model of CIA are currently underway in our laboratory and will be reported in the near future. +

AUTHORS INSIGHT ON THE TOPIC

Findings from clinical studies have implicated the uPAS in the pathogenesis of RA. Experimental evidence over the past 20 years has revealed similar pathologic roles for uPAS in common collagen-induced and other systemic mouse models of arthritis. In particular, recent evidence for the pharmacologic inhibition of uPA proteolytic activity has demonstrated the efficacy of this approach in pre-clinical models of RA. As such targeting of the uPAS represents an attractive and clinically relevant approach for the development of new RA drug candidates.

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CONFLICT OF INTEREST

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