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Research Article

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Elevated expression of TAM receptor tyrosine kinase in synovial fluid and synovial tissue of rheumatoid arthritis

Li Zheng[1,3,‡], Liling Xu [1,2,‡,*], Fanlei Hu[1,2], Jimeng Xue[1,2], Mingxin Bai[1,2], Ranran Yao[1,2], Huaqun Zhu[1,2], Hua Zhong[1,2], and Yin Su[1,2]

[1]Department of Rheumatology and Immunology, Peking University People's Hospital, Beijing, China
[2]Beijing Key Laboratory for Rheumatism Mechanism and Immune Diagnosis (BZ0135), Beijing, China
[3]Department of Rheumatology, The Second Hospital of Shanxi Medical University, Beijing, China

[‡]Li Zheng and Liling Xu contributed equally to this work.
[*]Correspondence: Yin Su, Department of Rheumatology and Immunology, Peking University People's Hospital, 11 Xizhimen South Street, Beijing 100044, China. Email: suyin0921@163.com

Abstract

To investigate the expression and roles of TAM (Tyro3/Axl/Mer) receptor tyrosine kinases (TK) in synovial fluid and synovial tissue of patients with rheumatoid arthritis (RA). The expression of TAM TKs in the synovial fluid and synovial tissues of RA and osteoarthritis (OA) patients was measured by ELISA and immunohistochemistry. The relationships between soluble TAM TKs (sTAM TKs) levels and the clinical features, laboratory parameters and disease activity were analyzed in RA. The concentrations of sTAM TK in the synovial fluids of RA patients were increased in comparison to those of OA patients. Compared with OA patients, the expression of membrane Tyro3 TK (mTyro3 TK) and mMer TK in RA patient synovial tissue were significantly increased, which may partly explain the possible mechanism of elevated levels of sTAM TK in RA patient synovial fluid. sAxl TK levels were decreased in RA patients under sulfasalazine treatment and elevated in patients under Iguratimod treatment. Furthermore, sTyro3 TK levels were positively correlated with erythrocyte sedimentation rate (ESR) and negatively correlated with white blood cells (WBCs), red blood cells (RBCs), and hemoglobin (HB) in RA patients. The levels of sMer TK were positively associated with disease duration and rheumatoid factor (RF) and negatively correlated with HB, complement 3 (C3), and C4. Taken together, TAM TKs might be involved in RA synovial tissue inflammation.

Keywords: rheumatoid arthritis, TAM receptor tyrosine kinase, synoviocytes, synovial fluid, clinical features

Abbreviations: ACR, American College of Rheumatology; ADAM, A disintegrin and metalloproteinase; anti-CCP antibody, anti-cyclic citrullinated peptide antibody; BSA, bovine serum albumin; C3, complement 3; CIA, collagen-induced arthritis; CRP, C-reactive protein; DAB, diaminobenzidine; DAS28-ESR, the 28-joint Disease Activity Score-erythrocyte sedimentation rate; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatology; FLSs, fibroblast-like synoviocytes, FNIII, fibronectin type III; Gas6, growth arrest-specific 6; HB, hemoglobin; HCQ, hydroxychloroquine; HCs, healthy controls; HRP, Horseradish peroxidase; Ig, immunoglobulin; IGU, iguratimod; IHC, immunohistochemistry; KRN STA, KRN serum transfer arthritis; LEF, leflunomide; MTX, methotrexate; mTyro3/Axl/Mer TK, membrane Tyro3/Axl/Mer TK; OA, osteoarthritis; PBS, phosphate-buffered saline; pSS, primary Sjogren's syndrome; RA, rheumatoid arthritis; RA, rheumatoid arthritis; RBCs, red blood cells; RF, rheumatoid factor; SASP, sulfasalazine; SD, standard deviation; SLE, systemic lupus erythematosus; sTyro3/Axl/Mer TK, soluble Tyro3/Axl/Mer TK; TAM TK, Tyro3/Axl/Mer receptor tyrosine kinases; TGT, tripterygium glycosides tablets; TMB, tetramethylbenzidine; WBCs, white blood cells.

Introduction

TAM receptors (Tyro3, Axl, and Mer) are a unique subfamily of receptor protein-tyrosine kinases that are composed of two immunoglobulin-like domains, two fibronectin type III (FNIII) domains, a transmembrane domain and a protein-tyrosine kinase domain [1]. TAM receptors are widely expressed in cells of the immune, nervous and reproductive systems, such as macrophages, dendritic cells, sertoli cells, and Schwann cells [2–4]. Previous studies have suggested that with binding to their cognate ligands, growth arrest-specific 6 (Gas6) or protein S, TAM receptors play pivotal roles in a gamut of diseases such as infection, chronic inflammatory conditions, and cancer [5]. Furthermore, the connection between the FNIII domains and the transmembrane domain of TAM receptors could be cleaved by metalloproteinases such as A disintegrin and metalloproteinase (ADAM) 10 and ADAM17 [6–8]. In recent years, studies have revealed that the soluble TAM receptors (sTyro3/sAxl/sMer TK) induced by the abovementioned proteolysis cleavage also have an essential function in the homeostatic regulation of many diseases and may even serve as a potential biomarker [9].

With increasing attention to TAM receptors, the roles of TAM receptors have been implicated in different autoimmune diseases [9]. TAM receptor knockout (KO) mice exhibited enhanced antigen-presenting cell activity and downregulated inflammatory immune response, accompanied by enlargement of the spleen and lymph nodes and infiltration of lymphocytes in tissues and organs [10]. In addition, compared with healthy

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controls (HCs), higher expression of membrane Mer TK (mMER TK) on dendritic cells was observed in systemic lupus erythematosus (SLE) patients, which may be induced by corticosteroids [4]. In an anti-GBM-induced lupus-like nephritis mouse model, treatment with Axl TK inhibitor could dampen glomerular proliferation, and improve kidney function [11]. A recent study by Lucrezia Rovati and his colleagues revealed that compared with that in pSS patients, the expression of mMer TK on macrophage in IgG4-related disease lesions was more abundant, which might be involved in the process of inflammation and tissue fibrosis [12].

Moreover, the association between the soluble form of TAM receptors and autoimmune disease also has been increasing attention. Both of our previous studies and other studies found that levels of sMer TK and sAxl TK were positively correlated with SLE patient disease activity [13–17]. And Qin et al. found that the plasma concentrations of sMer TK were elevated in primary Sjogren's syndrome (pSS) patients and also associated with patient disease activity [18]. And autoantibody against Tyro3 TK was significantly increased in SLE patients compared to HCs and sTyro3 TK levels were also positively correlated with disease activity [19]. Research on pregnant women with positive antiphospholipid antibodies also suggested that the concentrations of sAxl TK were elevated [20].

Rheumatoid arthritis (RA) is a systemic chronic disease characterized by synovitis and damage to both cartilage and bone in synovial joints, leading to functional disabilities that affect up to 1.8% of the population worldwide [21, 22]. The pathological process of synovitis, synovial hyperplasia with neovascularization, and the excess production of synovial fluid could lead to the destruction and erosion of articular cartilage [23]. Although the etiology of RA remains unclear, it is well established that both fibroblast-like synoviocytes (FLSs), T cells, and monocytes/macrophages contributed to synovial inflammation and joint destruction.

The negative regulatory roles of TAM receptor tyrosine kinases have been widely proved in RA. More than a decade ago, a study by Van den Brand et al found that overexpression of Gas6 by adenoviruses could significantly alleviate the symptoms in mice with collagen-induced arthritis (CIA) [24]. And results from Waterborg et al. showed that mAxl TK was expressed by M2-like macrophages in the ankle and knee synovial biopsies, which could dampen arthritis in ankle joints [25]. Further study revealed that lower levels of mAxl TK on $CD1c^+$ DCs may contribute to the pathogenesis of RA [24]. Consistently, it is reported that mMer TK may also play a protective role in joint inflammation of KRN serum transfer arthritis (KRN STA) [26]. And Zhang et al. also found that compared with RA patients, mMer TK on macrophages was more higher expressed in OA patients [27].

However, a recent study showed that even though TAM TK ko mice could develop bone marrow edema, no synovial inflammation nor bone destruction was found surprisingly [28]. Importantly, compared with wild-type mice, CIA mice induced by Tyro3 TK KO mice displayed less synovial hyperplasia, osteoclast numbers, and bone damage [29]. In addition, our previous study further indicated that elevated mTyro3 TK on $CD14^+$ $CD16^-$ monocytes may also serve as a critical signal for osteoclast differentiation in RA [30], and increased sTyro3 TK concentrations in RA patient sera were significantly associated with clinical features, disease activity and bone destruction [31]. Similarly, serum levels of sAxl in naive-to-treatment RA patients also showed a significant increase compared to patients in remission [24].

Taken together, these studies indicated that TAM receptors may be important molecules involved in the pathogenesis of RA. And opposing roles of TAM receptors in RA also attracted more and more attention. However, the functions of TAM receptors in RA synovial tissue and synovial fluid remain unclear and need to be further explored. In this study, the expression of TAM receptors in RA patients' synovial fluid and synovial tissue was detected. The relationships between the levels of TAM receptors and synovial inflammation, clinical features, laboratory indices, and disease activity were further explored to illumine their roles in the pathogenesis of RA.

Materials and methods

Patients and controls

RA patients ($n = 84$) and OA patients ($n = 54$) as disease controls were enrolled in this study by the Department of Rheumatology, Peking University People's Hospital, China. Patients with RA fulfilled the 1987 American College of Rheumatology (ACR) revised criteria or RA classification diagnostic criteria copublished by the 2010 ACR and the European League Against Rheumatology (EULAR) [32, 33]. OA patients met the 1995 American College of Rheumatology criteria [34]. All synovial fluid specimens from 70 RA patients and 40 OA patients were extracted using a sterile knee puncture, centrifuged at 1500 r/min for 10 min, and immediately stored at −80°C. Synovial tissues from 14 RA patients and 14 OA patients were collected from the most inflamed area during total knee replacement. Synovial tissues were directly put in the Petri dish containing phosphate-buffered saline (PBS) After surgical separation, then adipose tissue and connective tissue were removed. All tissues were fixed in 4% paraformaldehyde and were embedded in a paraffin block. Then the specimens were sliced into 4 μm sections and were analyzed by immunohistochemistry. This study was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital (2016PHB163-01). Written informed consent was obtained from all participants.

Clinical and laboratory parameters of RA patients

Demographic and clinical characteristics were collected, including sex, age, disease course, clinical features, and laboratory indices, such as white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), platelets (PLTs), D-dimer, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), immunoglobulin (Ig)G, IgA, IgM, rheumatoid factor (RF), and anti-cyclic citrullinated peptide antibody (anti-CCP antibody). RA disease activity was assessed by using the 28-joint Disease Activity Score-erythrocyte sedimentation rate (DAS28-ESR) based on the assessment of 28 joints and ESR. According to the recommendations from the European League Against Rheumatism (EULAR), 10 RA patients were under remission (DAS28-ESR < 2.6), 50 patients were under low or middle disease activity (2.6 < DAS28-ESR < 5.1), and 24 patients were under high disease activity (DAS28-ESR > 5.1).

The levels of sTAM TK in synovial fluid of RA and OA patients

The levels of sTAM TK in synovial fluid were detected using an enzyme-linked immunosorbent assay (ELISA). Sandwich ELISA kits for sTyro3 TK (DYC891), sAxl TK (DY154), and sMer TK (DYC6488) were obtained from R&D Systems (Minneapolis, MN, USA). Ninety-six-well plates (Corning, New York, NY, USA) were coated overnight with 100 µl capture antibody dilution of Tyro3/Axl/Mer TK. Next, the plates were washed with 300 µl 0.05% Tween 20 in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 nM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2–7.4, 0.2 µm filtered) three times. Then, the plates were blocked with 300 µl of 1% bovine serum albumin (BSA) in PBS for 1.5 h. The plates were washed as mentioned above every two steps. A seven-point standard curve was made by 2-fold serial dilution of recombinant proteins, and blank controls were reagent diluent alone. The synovial fluid samples of sAxl TK were diluted 100 times with blocking buffer, while no dilution was performed for sTyro3/sMer TK detection. Then, 100 µl samples and standards were added and incubated for 2 h at room temperature. After washing the plates, 100 µl biotinylated goat anti-human Tyro3/Axl/Mer TK antibody dilutions were added and incubated for 2 h at room temperature. The plates were washed, and 100 µl/well streptavidin-conjugated horseradish peroxidase was added and incubated for 20 min. Then, the plates were washed, tetramethyl benzidine (TMB) (Neobioscience, China) was added as the substrate solution, and the color reaction was stopped by the addition of 50 µl/well 50 µM 2 N sulfuric acid. The absorbance was read at 450 nm with a correction wavelength set at 570 nm using a microplate reader (BioTek, Winooski, VT, USA). The levels of sTyro3/sAxl/sMer TK were calculated with all-in-one microplate reader software.

The expression of TAM TK in synovial tissue in patients with RA and OA

The expression of Tyro3 TK, Axl TK, and Mer TK in synovial tissue of all patients was detected by immunohistochemistry (IHC). The thickness of each synovial tissue paraffin section was 4 µm. Rabbit monoclonal IgG against human Tyro3 TK (catalog no.: ab109231, dilutions: 1:400, Abcam, UK), Axl TK (catalog no.: ab219651, dilutions: 1:200, Abcam, UK), and Mer TK (catalog no.: ab52968; dilutions: 1:500, Abcam, UK) was used as the primary antibody. Rabbit IgG at the same concentration of anti-Axl/Mer/Tyro3 TK antibodies (catalog no.: ab172730, Abcam, UK) was used as a negative control. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology, China) was used as a secondary antibody. Sections were deparaffinized and rehydrated through xylene and a series of graded alcohols and washed in 0.01 mol/l PBS three times. Antigen retrieval was performed in Tris/EDTA buffer (pH 9.0) for Axl TK and Mer TK and 0.01 mol/l sodium citrate buffer solution (pH 6.0) for Tyro3 TK at 98°C. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. The primary antibody or Rabbit IgG (used for a negative control) was added to each section and incubated overnight at 4°C. After incubation, the slides were washed with PBS, incubated with secondary antibody for 30 min at room temperature in a wetting box, and then washed with PBS repeatedly. 3,3′-Diaminobenzidine (DAB) was used for staining lasting for 1.5–2 min and ended by washing with PBS. Then the sections were counterstained with hematoxylin, which was ended by washing with PBS when the nucleus turns blue. The slides were dehydrated with sequential ethanol starting with 70%, followed by 80%, 95%, and 100% for 20 s, and then sealed finally.

All slides were observed using a microscope (LEICA DM4000B, Germany) by three professional pathologists with a double-blinded method. Three tissue sections per patient were analyzed in all fields. According to the degree of staining, the positive signals were classified into 4°C: 0: no staining; 1: weak staining; 2: moderate staining; and 3: yellow to brownish-yellow staining. According to the proportion of positive cells, the positive degrees were divided into 4 °C were further defined: 0: no staining; 1: <10% staining; 3: 10–50% staining; and 4: >50% staining. The average of the above two scores was calculated.

Statistical analysis

All analyses were performed using SPSS version 25.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are expressed as the means ± SD or median with a range (P25, P75). Statistical differences in each group were calculated with a one-way analysis of variance (namely *F* test, the test statistic of the method is called *F*), Student's *t*-test (normal distribution), or Mann–Whitney *U* (abnormal distribution, the test statistic of the method is called *Z*). Correlations were evaluated with Pearson's rank correlation (normal distribution) or Spearman's rank correlation (abnormal distribution) test. A *P* value less than 0.05 was considered significant.

Results

Demographic and clinical characteristics of RA patients

Eighty-four RA patients were enrolled in the present study, of whom 70 patients were enrolled for synovial fluid samples for ELISA and 14 patients were enrolled for synovial tissues for immunohistochemistry staining. The ratio of females to males was 6:1 (72 females and 12 males), the age range was 22–85 years old, and the median age was 58.5 years old. The median duration was 8 years (IQR = 3–18). The median level of anti-CCP antibody was 168.2 IU/ml (IQR = 64.3–214.1 IU/ml) and the mean DAS28-ESR score was 4.34 ± 1.42. The demographic and clinical characteristics of the RA patients are shown in Table 1.

Increased levels of sTAM TK in the synovial fluid of RA patients

According to the DAS28-ESR score of 70 RA patients enrolled for synovial fluid, 8 patients were under remission, 36 were under low or middle disease activity, and 26 patients were under high disease activity. At first, we detected the levels of sTAM TKs in the synovial fluid of RA and OA patients. The results showed that RA and OA patients displayed the same concentration patterns of sTAM receptors in synovial fluid: sAxl TK > sMer TK > sTyro3 TK (RA: $F = 126.72$, $P < 0.001$; OA: $F = 170.37$, $P < 0.001$) (Fig. 1a and b).

Moreover, we compared the levels of sTAM TK between RA and OA patients and found that the concentrations of sTyro3 TK, sAxl TK, and sMer TK in RA patient synovial fluid (sTyro3 TK 1.2 ng/ml, IQR = 1–1.5 ng/ml; sAxl TK: 35.9 ng/ml, IQR = 25.8–47.1 ng/ml; sMer TK:5.8 ± 2.1 ng/ml)

were significantly increased comparing with OA patients (sTyro3 TK: 0.8 ± 0.3 ng/ml; sAxl TK: 18.5 ± 8.3 ng/ml; sMer TK: 1.4 ng/ml, IQR = 0.9–2.3 ng/ml) (sTyro3 TK: $Z = -5.686$, $P < 0.001$; sAxl TK: −6.583, $P < 0.001$; sMer TK: $Z = -82$, $P < 0.001$) (Fig. 2a-c).

Higher expression of mTyro3 TK and mMer TK in synovial tissue of RA patients

Then, to further explore the possible mechanism of elevated levels of sTAM TK in RA patient synovial fluid, we detected the expression of mTAM TK in synovial tissue of RA and OA patients. Fourteen RA patients and 14 OA patients were enrolled. And 4 RA patients were under remission and 10 were under low or middle disease activity. We found that compared with OA patients (4.85, IQR = 3.25–9.0), the expression of mTyro3 TK in RA patients (10, IQR = 7.6–11.3) was significantly increased ($Z = -2.235$, $P = 0.025$) (Fig. 3a and b). Furthermore, the expression of mMer TK was also elevated in synovial tissue in RA patients (6.5, IQR = 3.9–7.5) in comparison to OA patients (3.7, IQR = 2.2–4.7) ($Z = -2.387$, $P = 0.015$) (Fig. 3c and d). However, there was no significant difference in the expression of mAxl TK between RA (4.5 ± 3.0) and OA patients (3.5, IQR = 0.7–9.0) ($Z = 0.677$, $P = 0.684$) (Fig. 3e-f).

Table 1: Demographic and clinical characteristics of RA patients

Characteristics	RA patients ($n = 84$)
Age, years	58.5 (48–64)
Female (*n*, %)	72, 85.7%
Male (*n*, %)	12, 14.3%
Duration of RA (years)	8 (3–18)
SJC (0–28)	2.5 (1–8)
TJC (0–28)	2.5 (1–10)
RF (IU/ml)	58.6 (20–257)
RF positive (*n*, %)	61, 72.6%
Anti-CCP antibody (IU/ml)	168.2 (64.3–214.1)
Anti-CCP antibody positive (*n*, %)	73, 86.9%
ESR (mm/h)	45.5 (20–68.8)
CRP (mg/l)	24.8 (6.05–52)
DAS28-ESR	4.34 ± 1.42
Under treatment (*n*, %)	
NASIDs	16,19%
Corticosteroids	23,27.4%
MTX	26,30.9%
LEF	40,47.6%
SASP	15,17.9%
HCQ	17,20.2%
IGU	9,10.7%
TGT	8,9.5%
TNF-α inhibitor	5,5.9%

RA, rheumatoid arthritis; SJC, swollen joint count; TJC, tender joint count; RF, rheumatoid factor; Anti-CCP antibody, anti-cyclic citrullinated peptide antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score 28. NSAIDs: nonsteroidal anti-inflammatory drugs; MTX: methotrexate; LEF: leflunomide; SASP: sulfasalazine; HCQ: hydroxychloroquine; IGU: iguratimod; TGT: tripterygium glycosides tablets.

Effect of different treatments on synovial fluid levels of sTAM TK in RA patients

Next, we also evaluated whether the treatment has effects on the sTAM levels in synovial fluid of RA patients. The patients were divided into two groups respectively according to each ongoing treatment, including NSAIDs, corticosteroids, methotrexate (MTX), leflunomide (LEF), sulfasalazine (SASP), hydroxychloroquine (HCQ), Iguratimod (IGU), tripterygium glycosides tablets (TGT), and TNF-α inhibitor. Surprisingly, the results suggested levels of sAxl TK were lower in RA patients under SASP treatment ($Z = -3.202$, $P = 0.001$) compared to those without SASP treatment (Fig. 4a). While as against patients without IGU treatment, higher levels of sAxl TK were found in patients under IGU treatment ($Z = -2.418$, $P = 0.016$) (Fig. 4b). Comparing with patients without TNF-α inhibitor treatment, sMer TK concentrations showed an upward trend but had no significance ($t = 2.332$, $P = 0.055$) (Fig. 4c). No difference was observed between distinct undergoing treatment groups on sTyro3 TK.

The correlation between the expression of sTAM TK and parameters of RA patients

Then, we analyzed the relationship between synovial fluid levels of sTAM TK and clinical features in RA patients. Firstly, there was no difference between female and male patients, seropositive and seronegative patients on sTAM TK levels. while the concentration of sTyro3 TK was positively and significantly correlated with ESR ($r = 0.309$, $P = 0.009$) (Fig. 5a) but negatively correlated with WBC ($r = -0.257$, $P = 0.037$), RBC ($r = -0.347$, $P = 0.004$), and HB ($r = -0.366$, $P = 0.02$)

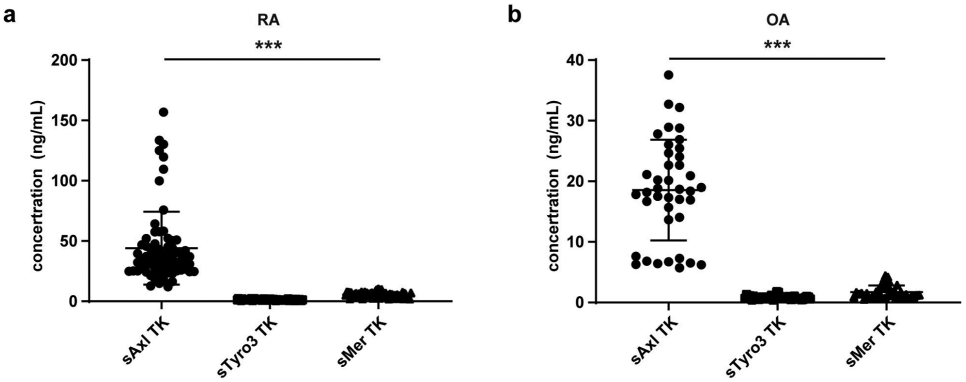

Figure 1: The expression patterns of soluble TAM receptors in synovial fluid from RA (a) and OA (b) patients. RA and OA patients displayed the same expression patterns of soluble TAM receptors in synovial fluid: sAxl TK > sMer TK > sTyro3 TK. *** $P < 0.001$.

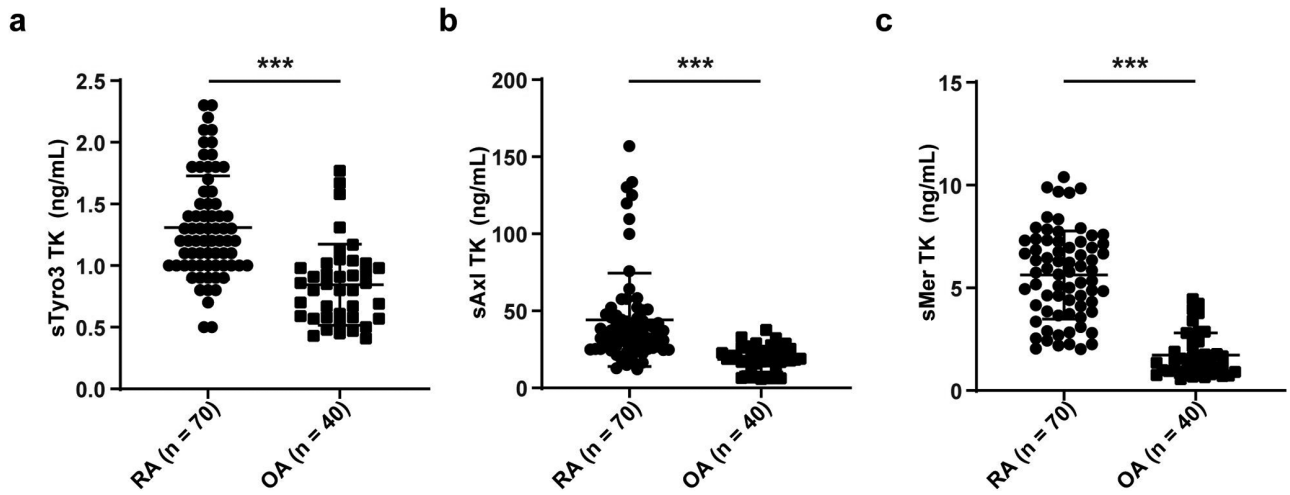

Figure 2: sTyro3 TK, sAxl TK and sMer TK levels in synovial fluids of RA and OA patients. Compared with OA patients, the levels of sTyro3 TK (a), sAxl TK (b) and sMer TK (c) in RA patient synovial fluids were significantly increased. ***$P < 0.001$.

(Fig. 5b–d). However, there was no correlation between the levels of sTyro3 TK and age, duration, PLT, C3, C4, IgA, IgG, IgM, CRP, RF, anti-CCP antibody, swollen joint counts (SJC), or tender joint counts (TJC).

Furthermore, there were positive associations between the levels of sMer TK and disease duration ($r = 0.399$, $P = 0.001$) and RF ($r = 0.326$, $P = 0.006$) in RA patients (Fig. 5e and f). There were significant negative correlations between the concentrations of sMer TK and HB ($r = -0.317$, $P = 0.007$), C3 ($r = -0.427$, $P = 0.000$), and C4 ($r = -0.532$, $P = 0.000$) (Fig. 5g–i). However, the concentrations of sAxl TK showed a positive correlation with RBCs ($r = 0.347$, $P = 0.004$), HB ($r = 0.29$, $P = 0.015$) and PLT ($r = 0.315$, $P = 0.008$) (Fig. 5J–l). No correlation was found between sMer TK/sAxl TK and other clinical parameters in RA patients.

To further illustrate the relationship between the levels of sTAM TK and RA patient disease activity, we calculated the DAS28 score of 70 RA patients. However, the results showed that there was no significant correlation between sTyro3 TK ($r = 0.095$, $P = 0.435$), sAxl TK ($r = -0.007$, $P = 0.954$), sMer TK ($r = 0.069$, $P = 0.569$) and the DAS28-ESR score.

Discussion

Classically, TAM TKs and their ligands act as pleiotropic inhibitors of the innate inflammatory response [5]. Data from *in vitro* experiments provided direct evidence that mTAM TKs are significantly upregulated in dendritic cells (DCs) as a consequence of Toll-like receptors (TLRs) engagement [35]. In addition, the mechanism of TAM TKs involves upregulation of the suppressor of cytokine signaling proteins SOCS1 and SOCS3 [35]. These SOCS E3 ubiquitin ligases are responsible for pleiotropic downregulation of the immune response through the turnover of molecules that function in critical, positive regulatory signaling cascades, such as the TLR, NF-κB and JAK-STAT pathways [36]. An additional mechanism of TAM TKs mediated inhibition of inflammation includes the upregulation of the transcription factor twist, leading to downregulation of TNF-α in turn [37].

In recent years, deficiencies in TAM TKs have been thought to a participant in chronic inflammatory and autoimmune diseases [38]. Therefore, we hypothesized that TAM receptors are closely related to the pathogenesis of RA. While the current opinion of TAM TKs on RA still exist some contradictions. Some studies indicated that Axl TK and Mer TK may play a protective role in RA [24–26], but our previous data and van den Brand BT's study showed that Tyro3 TK may contribute to the pathogenesis of RA via aggravating inflammatory and joint destruction [29–31].

It has been demonstrated that the inflammatory synovial fluid microenvironment significantly mediates cell death and apoptosis of human chondrocytes and triggers them to actively take part in inflammatory processes in rheumatic joint diseases by differential secretion of various specific cytokines [39]. Ruiz-Heiland et al. found that Tyro3-Gas6 is a critical signal for synovial hyperplasia and joint destruction in the murine arthritis model [29]. However, less is known about the expression and function of mTAM TKs in RA patient synovial tissues, which act as the main inflammatory site of RA. Therefore, to fully explore the roles of TAM TKs in RA, we further systematically revealed the expression of TAM TKs in synovial fluid and synovium in RA patients.

Firstly, we noticed that sTAM receptors in synovial fluid of RA and OA patients showed the same expression patterns: sAxl TK > sMer TK > sTyro3 TK compared with our previous study on sTAM TK in RA patient serum [31] and study from Julia Vullings et al [40]. However, Julia Vullings et al. reported that both sTyro3 TK and sMer TK levels in RA patient synovial fluid were elevated compared to those in OA patients, and no significant differences in sAxl were observed [40]. In our study, with expanded sample size, we found that compared with OA patients, sTyro3 TK, sMer TK and sAxl TK levels were significantly increased in the synovial fluids of RA patients, which may better reflect the expression of sTAM TKs in synovial fluid of RA and OA. Interestingly, we also noticed that the levels of sMer TK in patients enrolled in our study were significantly lower than those in patients included in Julia Vullings's study. The above differences may be due to the different expression profiles of different populations, which need to be verified with a larger sample size.

To further explore the possible mechanism of elevated levels of sTAM TKs in RA patient synovial fluid, we further elaborated on the expression of mTAM TKs in synovial tissue of RA and OA patients. The results showed that the expression

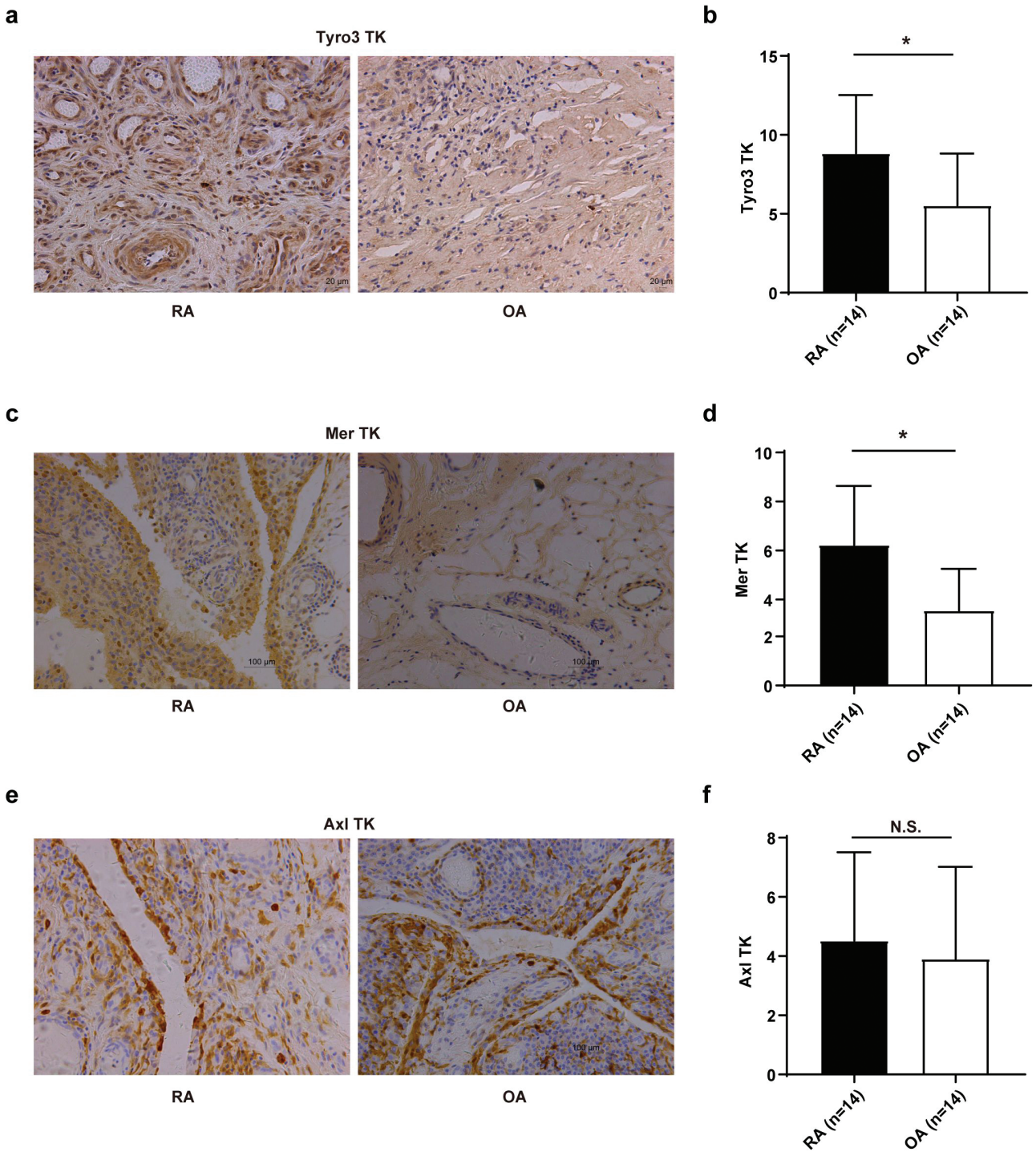

Figure 3: The expression of TAM TK in synovial tissue of RA and OA patients. (a) Representative Tyro3 TK expression in synovial tissue of RA (left) and OA (right) patients is shown. (b) The statistical results of the final comprehensive score of Tyro3 TK between RA and OA patients. (c) Representative experiment of Mer TK expression in synovial tissue of RA (left) and OA (right) patients is shown. (d) The statistical results of the final comprehensive score of Mer TK between RA and OA patients. (e) Representative experiment of Axl TK expression in synovial tissue of RA (left) and OA (right) patients is shown. (f) The statistical results of the final comprehensive Axl TK score between RA and OA patients. *$P < 0.05$. N.S. = no significance.

of mTyro3 TK and mMer TK was elevated in synovial tissue in RA patients in comparison to OA patients, while there was no significant difference in the expression of mAxl TK. Thus, it may partly explain the increase of sTyro3 TK and sMer TK in RA patient synovial fluid. At first, it has been reported that proinflammatory monocytes may cause increased concentrations of metalloproteinases, which would advance the proteolytic cleavage of the extracellular part of TAM TKs. Secondly, the accumulation of apoptotic cells could activate the liver X receptor, and cell-activating substances such as LPS have been shown to induce the expression and cleavage of TAM TKs [41]. Moreover, we noticed that a similar compartmentalized expression of TAM TKs was found in a study by Vullings et al. and us. Whereas no difference in all three TAM

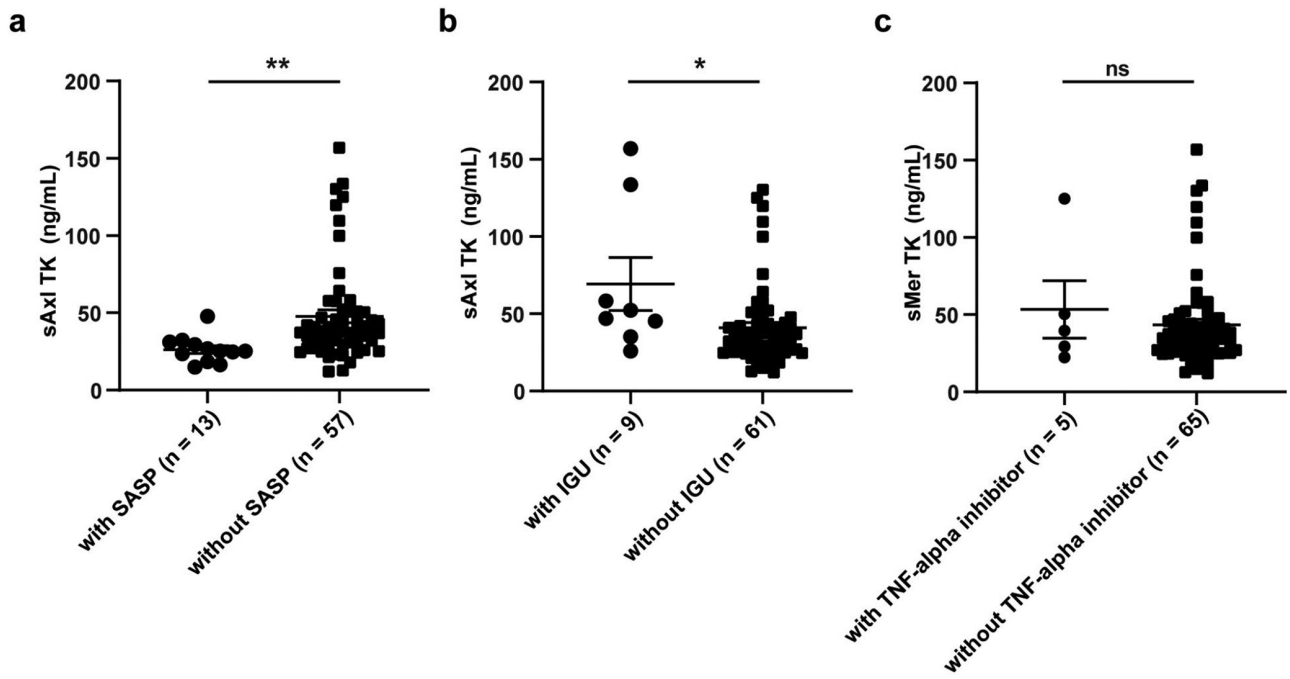

Figure 4: Effect of different treatment on synovial fluid levels of sTAM TK in RA patients. (a) sAxl TK levels were lower in 13 RA patients under sulfasalazine treatment compared to 57 patients without SASP treatment. (b) Higher levels of sAxl TK were found in 9 patients under IGU treatment. (c) Compared with 65 patients without TNF-α inhibitor treatment, sMer TK concentrations showed an upward trend in 5 patients with TNF-α inhibitor treatment but had no significance. SASP: sulfasalazine; IGU: Iguratimod. $^{**}P < 0.01$, $^{*}P < 0.05$. ns = no significance.

TKs was found between RA and OA patients in Vulling's research. Presumably, it is because of the small samples with only 2 RA patients and 8 OA patients.

In addition to the relationship with the cleavage of metalloproteinases, it has been reported that medical treatment may also influence TAM TK expression. Axl TK and Mer TK expression on macrophages could be induced in mice treated with dexamethasone [42]. Monocytes/macrophages from COPD patients and SLE patients treated with corticosteroids also displayed higher Mer TK expression [4, 43]. Besides, the inhibitor role of anti-TNF agents on Mer TK was also reported in RA [44]. However, the role of medical treatment on sTAM TK is still unclear. So we collected the information about the ongoing treatment of RA patients and analyzed the difference serum levels of sTAM TKs between patients who were treated with or without the drug. The data showed the promoting effect of SASP and inhibiting role of IGU on sAxl TK levels. Even though the reason remains obscure, the results may provide some clues for further exploring the potential mechanism.

Interestingly, a study found that by interacting with Gas6, Axl TK has mitogenic and survival activities for NIH3T3 fibroblasts [45]. The research from Loeser et al. also suggested a role of Tyro3 TK in the survival of cells involved in synovial inflammation, such as fibroblasts, monocytes/macrophages, endothelial cells, and chondrocytes [46]. Therefore, we preliminarily analyzed the expression profile of TAM TKs in synovial tissue according to cell morphology by two experienced professional clinic pathologists. The results showed that mTyro3 TK and mMer TK were predominantly expressed on synovial fibroblast-like cells, macrophages, lymphocytes, plasma cells, and endothelial cells in the synovial membrane lining layer of RA patients, which was basically consistent with the data from Julia Vullings et al. An earlier study by Waterborg CEJ and colleagues suggested the synovial lining layer cells in ankle joints of C57BL/6 mice were positive for the Axl TK [25]. We also found that mAxl TK was mainly expressed on vascular endothelial cells in synovial tissue. These results may indicate that the three members of TAM TKs may play different roles in RA. Moreover, over the last decade, numerous publications have shown that the synovial tissue is a very heterogeneous entity, different synovial histological "pathotypes" can be identified and correlated with clinical parameters and disease activity [47]. However, further study is still needed to confirm which cells could express mTAM receptors by co-staining with specific markers of these cells and the expression of mTAM receptors in different synovial histological "pathotypes", which may be crucial for explaining the role of TAM TK in the synovial tissue of RA.

In the present study, we also found that the concentrations of sTyro3 TK were positively correlated with ESR and negatively correlated with WBC, RBC, and HB in RA patients. In addition, the levels of sMer TK were positively associated with disease duration and RF and negatively correlated with HB, C3, and C4. Consistent with most plasma results, it may better reflect the relationship between local TAM expression and joint inflammation. Our previous study showed that elevated sTyro3 TK levels in RA patient sera were significantly associated with clinical features, disease activity, and bone destruction [31], while no correlation was found between sTAM TKs and DAS28-ESR in this study. The synovial fluids used in this study were mainly collected during outpatient and treatment, while the serum samples used in our previous study were mainly collected during inpatient. The different disease activity and drug treatment might potentially induce the discrepancy. Nevertheless, the synovial sTyro3 was also found to be correlated with RA patient clinical features, such as ESR, Hb,

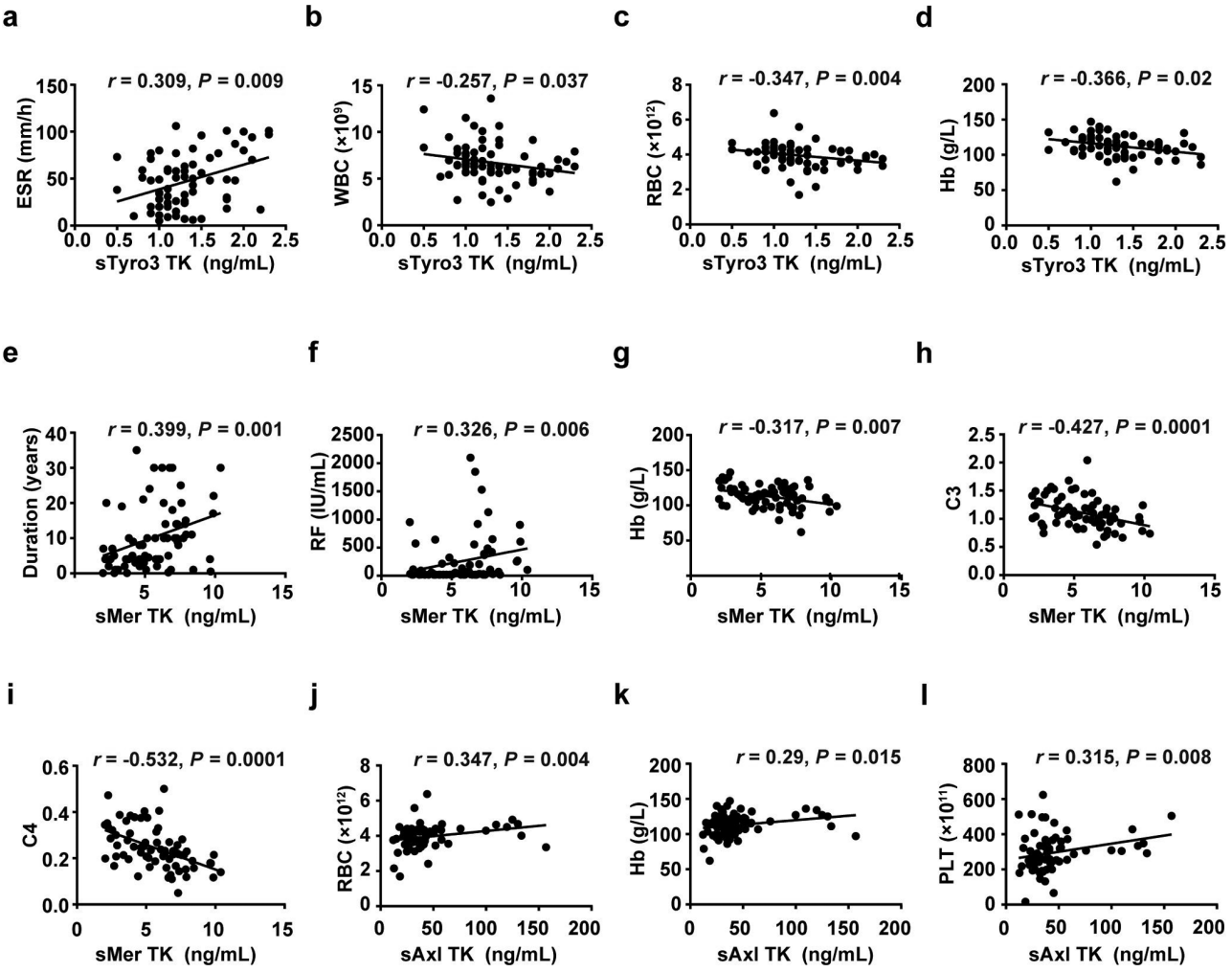

Figure 5: Correlations between sTAM TK levels in synovial fluid and RA patient clinical features. The sTyro3 TK levels were positively correlated with ESR (a) and negatively correlated with WBC (b), RBC (c) and Hb (d). Moreover, the levels of sMer TK were positively associated with disease duration (e) and RF (f) and negatively correlated with HB (g), C3 (h) and C4 (i). However, the concentrations of sAxl TK showed a positive correlation with RBC (j), Hb (k) and PLT (l).

WBC, et al. Considering the current findings and clinical application, we speculate that serum sTyro3 TK might be a more sensitive biomarker for RA.

The main limitation of our study was lacking functional validation and mechanism studies. A more in-depth study on the mechanism of TAM receptors in RA needs to be further performed. We have been studying the functions and mechanisms of TAM receptors in RA fibroblast-like synoviocytes. Hopefully, this may further elucidate the role of TAM receptors in the synovial pathogenesis of RA.

Conclusion

In summary, we systematically measured the levels of the three members of TAM TK subfamily in the synovial fluid and synovium of RA patients and simultaneously revealed their correlation with RA clinical features. The levels of sTyro3/Axl/Mer TK in the synovial fluids of RA patients were significantly increased compared to OA patients, similar to the expression of mTyro3/mMer TK in synovial tissue. These findings might further suggest the potential role of TAM receptors in the pathogenesis of RA.

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Ethics Approval

This study was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital (2016PHB163-01). Written informed consent was obtained from all participants.

Conflict of Interest

The authors declare that they have no competing interests.

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Author Contributions

LZ and LX performed the experiments, analyzed the data, and prepared the manuscript. YS conceived and designed the study, reviewed, and edited the manuscript. FH, JX, MB, RY, HZ, and HZ contributed to reagents/materials/analysis.

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