The level of synovial AXL expression determines the outcome of inflammatory arthritis, possibly depending on the upstream role of TGF-β1

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Abstract

Objective. To investigate the role of AXL, a member of the anti-inflammatory TYRO3, AXL MER (TAM) receptor family, in arthritis.

Methods. KRN serum transfer arthritis was induced in Axl−/− and wild-type mice. Knee and ankle joints were scored macro- and microscopically. Synovial gene and protein expression of Axl was determined in naïve and TGF-β1-overexpressing joints. AXL expression was determined in M1-like or M2-like macrophages and RA synovium. Human macrophages, fibroblasts and synovial micromasses were stimulated with TGF-β1 or the AXL inhibitor R428.

Results. Ankle joints of Axl−/− mice showed exacerbated arthritis pathology, whereas no effect of Axl gene deletion was observed on gonarthrosis pathology. To explain this spatial difference, we examined the synovium of naïve mice. In contrast to the knee, the ankle synovial cells prominently expressed AXL. Moreover, the M2-like macrophage phenotype was the dominant cell type in the naïve ankle joint. Human M2-like macrophages expressed higher levels of AXL and blocking AXL increased their inflammatory response. In the murine ankle synovium, gene expression of Tgfb1 was increased and Tgb1 correlated with Axl. Moreover, TGFB1 and AXL expression also correlated in human RA synovium. In human macrophages and synovial micromasses, TGF-β1 enhanced AXL expression. Moreover, TGF-β1 overexpression in naïve murine knee joints induced synovial AXL expression.

Conclusion. Differences in synovial AXL expression are in accordance with the observation that AXL dampens arthritis in ankle, but not in knee joints. We provide evidence that the local differences in AXL expression could be due to TGF-β1, and suggest similar pathways operate in RA synovium.

Key words: rheumatoid arthritis (RA), AXL, TGF-β1, inflammation, joint-specificity

Rheumatology key messages

- AXL is expressed on murine synovial lining cells of ankle, but not knee joints.
- TGF-β1 is a strong inducer of AXL on sentinel cells that occupy the synovium.
- AXL is a potential therapeutic target for RA, dependent on the level of expression.

Introduction

RA is a progressive and degenerative joint disease where chronic synovial inflammation and cartilage and bone destruction in multiple joints are the main hallmarks. Although often considered a disease of the adaptive immune system, innate immunity plays an important role in RA. This is highlighted by the fact that the number of synovial macrophages correlates with joint destruction in RA [1]. In addition, the efficacy of many biologicals is targeting cytokines mainly produced by macrophages [2].

One family of tyrosine kinase receptors expressed by macrophages and other sentinel cells, cells in the body’s first line of defence, are the TAM receptors. The TAM receptors – TYRO3, AXL and MER (gene name MERTK) – play a critical role in innate immune homeostasis [3–7]. The two principal TAM receptor ligands are Growth...
Arrest Specific 6 (GAS6) and Protein S (PROS1) [8–12]. Notably, GAS6 is a ligand for all three receptors but with the highest affinity for AXL. In contrast, PROS1 can only activate TYRO3 and MER but not AXL [10]. These ligands display divergent binding: they bridge TAM receptors and phosphatidylserine expressed on the surface of apoptotic cells [10, 11]. Activation of the TAM receptors reduces numerous inflammatory cytokines [4, 7, 13, 14]. Moreover, activation of AXL inhibits Nucleotide-binding domain Leucine-rich Repeat (NLRR) family pyrin domain containing 3 (NLPR3) inflammasome activation, thereby inhibiting the maturation of IL 1β [15, 16].

We have previously shown that targeted delivery of TAM receptor ligand genes Pros1 or Gas6 diminishes the arthritis pathology effectively [13]. Because all three receptors can be activated by GAS6 [10, 11], the endogenous role of AXL in arthritis is not fully understood. AXL and GAS6 are expressed in synovial tissue of RA patients [17]. In addition, a synovial fluid proteome study identified the soluble form of AXL in RA synovial fluid [18]. In contrast, the plasma levels of soluble AXL and GAS6 are significantly reduced in RA patients. Moreover, GAS6 levels are lower in erosive compared with non-erosive RA [19]. Furthermore, AXL expression was significantly reduced in CD1c+ dendritic cells of RA patients [14]. To understand the role of AXL in RA, mechanistic studies are needed. Therefore, we studied the effect of Axl deficiency in the KRN serum transfer model of arthritis (KRN STA), an inflammatory polyarthritis model driven by macrophages [20].

Methods

Patient material

RA synovial tissue was obtained during surgery from the Radboud University Medical Center or the Sint Maartenskliniek (both Nijmegen, the Netherlands). This material was considered surgery surplus material. For the Radboud University Medical Center, patients gave informed consent for the surgery, were informed and were able to decline the use of their material for research. According to Dutch law, informed consent was not necessary. In addition, patient material was anonymized and no patient data is available. For the Sint Maartenskliniek, patients gave written informed consent for the use of their material for research. The patient material was pseudonymized and patient demographic information is listed in Supplementary Table S1, available at Rheumatology online. There was no need for the approval by an ethical committee. Procedures were performed in accordance to the code of conduct for responsible use of human tissue in medical research (https://www.federa.org/code-goedgebruik). Tissue samples were fixed in formalin for histochemistry. Subsequently, red blood cells were lysed. Cells were put in culture with RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 mM pyruvate and penicillin/streptomycin (P/S). Medium was refreshed weekly. RAFLS were used between passage 2 and 4.

Human CD14+ monocytes

Whole blood was mixed with PBS 1.5% acid citrate-dextrose solution A 1 : 1. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Eindhoven, the Netherlands). CD14+ cells were isolated with the MagniSort Human CD14 Positive Selection Kit according to manufacturer’s protocol (Invitrogen, Landsmeer, the Netherlands) (purity >90%). CD14+ cells were used to generate macrophages or synovial micromasses. Healthy donors gave informed consent for the use of the blood (Sanquin Blood Bank, Nijmegen, the Netherlands).

Human synovial three-dimensional micromasses

Methods for the generation of micromasses, RAFLS and CD14+ cells were used with a ratio of 1 : 5 (200,000 RAFLS and 1×10^6 monocytes per micromass). Cells were dissolved in ice-cold Matrigel (Corning, Amsterdam, the Netherlands) and droplets were placed in culture plates coated with poly-(2-hydroxyethyl methacrylate) (Sigma-Aldrich (now Merck), Schiphol-Rijk, the Netherlands). After 30-min gelation at 37°C, RPMI, supplemented with 10% FCS, 1 mM pyruvate and P/S was added. After 7 days of culture, the formation of a synovial-like lining and the presence of CD68+ macrophages was confirmed on histology. Subsequently, micromasses were cultured in the absence or presence of 10 ng/ml recombinant human TGF-β1 (Biolegend, San Diego, CA, USA) for 3 weeks. Medium was refreshed twice weekly. After 3 weeks, micromasses were lysed in TRIzol (Sigma Aldrich) or put on ice for 60 min to reliquify Matrigel and use cells for flow cytometry analysis.

TGF-β1 stimulation of RAFLS

RAFLS were plated in 24-wells-plates with 0.5×10^6 cells/well in RPMI supplemented with 10% FCS, 1 mM pyruvate and P/S. After 1 day, cells were serum-starved for 24 h by culturing in RPMI supplemented with 2% FCS, 1 mM pyruvate and P/S before stimulation with 10 ng/ml human TGF-β1. After 6 h, medium was removed and TRIzol was added. For each donor, each condition was performed in triplicate and the average is depicted.

Human monocyte-derived macrophages (Mφs)

CD14+ cells were plated in 24 wells-plates with 0.5×10^6 cells/well, RPMI supplemented with 10% FCS, 1 mM pyruvate, P/S and 50 ng/ml GM-CSF (Prospec, Rehovot, Israel) or 20 ng/ml hM-CSF (R&D, Abingdon, UK), termed M1-like GM-CSF Mφs and M2-like M-CSF Mφs, respectively. Medium was refreshed at day 3, and cells were ready for further experiments at day 6/7 or lysed in

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TRIzol. For each donor, each condition was performed in triplicate and the average is depicted.

TGF-β1 stimulation of MΦs

M2-like M-CSF MΦs were serum-starved for 24 h by culturing in RPMI supplemented with 2% FCS, 1 mM pyruvate, P/S and 20 ng/ml hM-CSF before stimulation with 10 ng/ml human TGF-β1. After 6 h, medium was removed and TRIzol was added. After 24 h, cells were harvested for flow cytometry analysis. For each donor, each condition was performed in triplicate and the average is depicted.

AXL inhibition of M-CSF MΦs

M2-like M-CSF MΦs were treated with 1 μM R428 dihydrochloride (Axon Medchem, Groningen, the Netherlands) for 2 h before stimulation with 100 ng/ml E. coli lipopolysaccharide, CpG oligodeoxynucleotide 2006 or Pam3CSK4. After 24 h, supernatants were stored in −20°C for further analysis. For each donor, each condition was performed in triplicate and the average is depicted.

Flow cytometry

Cells were incubated with human Fc block (BD Pharmingen, San Jose, CA, USA), diluted in FACS buffer (PBS 5% FCS, 2 mM EDTA). Subsequently, surface marker expression was evaluated using the following antibodies: CD14-FITC (325603; Biolegend), CD68-PE (12-0689; eBioscience (now Thermo Fisher), Eindhoven, the Netherlands), AXL (#8661; Cell Signalling, Leiden, the Netherlands) and Anti-Rabbit IgG F(ab')2 Fragment-Alexa Fluor 647 Conjugate (#4414; Cell Signalling). Samples were measured on BD FACSCalibur (BD Biosciences, San Jose, CA, USA) and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

Measurement of IL-1β and TNF-α

IL-1β and TNF-α in supernatants was measured on a Bio-Plex 200 system using a magnetic bead-based multiplex immunoassay. Data analysis was performed with Bio-Plex Manager software (both Bio-Rad, Lunteren, the Netherlands).

Human GAS6 ELISA

Supernatants were examined for GAS6 using the human GAS6 DuoSet ELISA (R&D) according to manufacturer’s instructions.
Mice

The Axl\(^{-/-}\) strain was generated as described previously [21]. All lines were backcrossed for >9 generations to a C57BL/6 background. For the KRN STA model, and histological and immunohistochemical analysis of AXL before the induction of this model, Axl\(^{-/-}\) and their wild-type (WT) littermates of this strain were used. Naive C57BL/6 mice were used for adenoviral overexpression study and for gene expression analysis of ankle and knee synovium. All mice were fed a standard diet with freely available food and water. Histological and immunohistochemical analysis were performed in a randomized and blinded manner. Clinical signs of arthritis were monitored macroscopically as described in the ‘Induction of KRN STA’ section. In vivo studies performed in the Netherlands complied with Dutch legislation and were approved by local authorities. In vivo studies executed in the USA were conducted according to guidelines established by the Salk Institutional Animal Care and Use Committee. For details, see Supplementary Table S2, available at Rheumatology online.

Induction of KRN STA

Arthritis was induced by two intraperitoneal injections, at day 0 and 2, of 150 \(\mu L\) arthritic K/BxN serum. Clinical signs of arthritis were monitored and macroscopically scored until the end of the experiment at day 7. Cumulative scoring was based on redness, swelling and ankylosis: 0 = no changes; 0.25 = 1-2 toes red or swollen; 0.5 = 3-5 toes red or swollen; 0.5 = swollen ankle; 0.5 = swollen footpad; 0.5 = severe swelling and ankylosis, with a maximal score of 2 per paw. Clinical signs of arthritis in knee joints were macroscopically scored at day 7 as determined by swelling and blood vessel formation, with a maximal score of 2 per knee joint.

Adenoviral overexpression

First generation adenoviruses encoding for luciferase (Ad Luc) and active porcine TGFB1 (Ad TGF-\(\beta\)1) (gift from Dr C.D. Richards, McMaster University, Canada) were produced as described before [13]. The overexpression of luciferase or TGFB1 was accomplished by an intra-articular injection into the knee joint of \(1 \times 10^7\) plaque-forming units.

Histological analysis

Human synovial biopsies were embedded in paraffin. Whole ankle and knee joints of mice were dissected, fixed in formalin, decalcified with 5% formic acid and embedded in paraffin. Tissue sections (7 \(\mu m\)) were stained with haematoxylin and eosin (both Merck, Schiphol-Rijk,

![Fig. 2 AXL protein expression in naive ankle and knee joints](https://academic.oup.com/rheumatology/article/58/5/596/629456)
the Netherlands) or with safranin O and fast green (both BDH Chemicals (part of Merck), Schiphol-Rijk, the Netherlands). Three semi-serial sections per joint were scored for histopathologic changes on a scale from 0/C150 by two independent observers in a blinded manner as described previously [22]. The average of this score was calculated, the average of two joints per mouse was calculated and is depicted. In knee joints, inflammation was determined by the presence of synovial cell infiltrates and inflammatory cell exudates. In ankle joints, this distinction could not be made because the joint space was too narrow and the infiltrate and exudate appear to merge into one another. Therefore, total inflammatory cell content was scored. Connective tissue damage was determined by the depletion of cartilage proteoglycan and by cartilage and bone erosion.

Immunohistochemistry

Protein expression of murine AXL, F4/80 and CD206 was evaluated on sections of ankle and knee joints. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked by 3% hydrogen peroxide in methanol. For AXL and CD206, antigen retrieval was performed in citrate buffer heated to 90°C. For F4/80, antigen retrieval was performed in citrate buffer heated to 37°C and subsequent trypsin treatment. For AXL, tissue sections were incubated with goat anti-AXL (AF854; R&D) or goat IgG (AB-108-C; R&D) followed by incubation with biotinylated anti-goat (BA-5000; Vector Laboratories, Peterborough, UK). For F4/80, tissue sections were incubated with rat anti-F4/80 (14-4801-81; Thermo Fisher, Eindhoven, the Netherlands) or rat IgG2a (553927; BD Pharmingen) followed by incubation with biotinylated anti-rat (BA-4001; Vector Laboratories). For CD206, tissue sections were incubated with goat anti-CD206 (AF2535; R&D) or goat IgG (AB-108-C) followed by incubation with biotinylated anti-goat (BA-5000). A biotin-streptavidin detection system was used according to manufacturer’s protocol (PK-6101; Vector Laboratories) and bound complexes were visualized with diaminobenzidine (Sigma Aldrich). Protein expression of human CD68 and CD163 was evaluated on synovial micromasses. Sections were deparaffinized and rehydrated.
Endogenous peroxidase was blocked by 3% hydrogen peroxide and antigen-retrieval was performed in citrate buffer. For CD68, tissue sections were incubated with mouse anti-CD68 (M0814, DAKO, Santa Clara, CA, USA) or mouse IgG (X0931, DAKO) followed by incubation with HRP-conjugated rabbit-anti-mouse Ig (P0260, DAKO). For CD163, tissue sections were incubated with mouse anti-human CD163 (MA5-11458; Thermo Fisher) or mouse IgG (X0931) followed by incubation with biotinylated anti-mouse IgG (BA-2001, Vector Laboratories). A biotin-streptavidin detection system was used according to manufacturer’s protocol (PK-6101). Bound complexes were visualized with diaminobenzidine. All sections were counterstained with haematoxylin.

RNA isolation and quantitative PCR
All tissues were disrupted using the MagNA Lyser (Roche) and lysed with TRIzol. Total RNA was extracted using TRIzol/Chloroform and treated with DNase followed by reverse transcription into cDNA using oligo(dT) primers. Quantitative PCR was performed with SYBR green PCR master mix using the StepOnePlus Real-Time PCR System (Applied Biosystems, Fostercity, CA, USA). The threshold cycle of the gene of interest was corrected for the reference gene Gapdh or GAPDH to obtain the $\Delta \Delta C_T$. The $C_T$ value was set to a threshold of 40 in samples in which no $C_T$ value was detected. The primer sequences are listed in Supplementary Table S3, available at Rheumatology online.

Statistics
Data were analysed with GraphPad Prism software (version 5.03). Data were checked for normality with the D’Agostino-Pearson omnibus test. Analysis of significance was performed using an unpaired $t$ test when data were normally distributed or a Mann-Whitney test if data were not normally distributed. Analysis of significance was performed using a paired $t$ test when data were paired. For correlations, the Pearson’s correlation coefficient was calculated. For evaluation of the macroscopic arthritis severity over time, analysis of significance was tested using a repeated measures two-way ANOVA.
Results

Arthritic Axl-/- mice show enhanced joint pathology in ankle joints but not in knee joints

To study the role of AXL in arthritis, KRN STA was induced in mice deficient for Axl and their WT littermates. At day 4 until the end of the experiment, Axl-/− mice showed a significantly higher macroscopic arthritis score in their hind paws relative to WT mice (Fig. 1A). Histological analysis showed that ankle joints in Axl-/− mice had exacerbated arthritis pathology (Fig. 1B, C). Knee joints of Axl-deficient mice showed, however, comparable clinical and histological arthritis scores to WT (Supplementary Fig. S1, available at Rheumatology online). To rule out that Axl deficiency led to aberrant ankle joints, we examined naive ankle joints of WT and Axl-/− mice. Histological analyses showed that Axl-/- mice neither spontaneously developed arthritis nor developed anomalous joints (Supplementary Fig. S2, available at Rheumatology online).

AXL is expressed by the synovial lining layer in ankle joints but not in knee joints

Due to the prominent differences between ankles and knees regarding Axl involvement in arthritis, we determined the synovial AXL expression in both joints in naive mice. The cells of the synovial lining layer of ankle joints were positive for the AXL tyrosine kinase receptor (Fig. 2A, B), whereas the synovial cells of the knee joints were completely devoid of AXL (Fig. 2C, D). The specificity of the staining was confirmed by joints from Axl-/- mice (Fig. 2A, C).

M2-like Mφs express enhanced levels of AXL, which controls their activation

As TAM receptors are expressed by sentinel cells, we further characterized the phenotype of the synovial macrophages in naive mice. Classically M1 and alternatively M2 activated macrophages are, among others, characterized by their expression of Nos2 and Arg1, respectively [23]. As shown in Fig. 3A, cells in the ankle appeared to have a more prominent M2-like phenotype as the ratio Arg1/Nos2 was significantly enhanced. Moreover, the synovial lining of the ankle joints was also markedly positive for F4/80, a macrophage marker, and CD206 (mannose receptor), another M2 marker (Fig. 3B). When we differentiated human blood monocytes to either M1-like GM-CSF or M2-like M-CSF Mφs, the latter cell type also expressed significantly higher levels of AXL and GAS6 whereas MERTK was unaltered (Fig. 3C). Furthermore, addition of the AXL inhibitor R428 led to a significant increase of IL-1β upon Toll-like
receptor ligation (Fig. 3D). In contrast, TNF-α and GAS6 levels were unaltered by AXL inhibition (Supplementary Fig. S3, available at Rheumatology online). The mouse in vivo and human in vitro data strongly suggest that AXL is expressed by M2-like macrophages in the murine ankle joint.

Synovial AXL expression correlates with TGFB1 expression

TGF-β1 potently stimulates M2-like macrophage differentiation [24, 25] and enhances AXL expression in Langerhans cells [26] and cancer cells [27]. Therefore, we examined the synovial expression of several TGF-β genes in ankle and knee joints. Tgfb1, and also Tgfb2 expression, were significantly higher in naïve murine ankle joints compared with knee joints, whereas Tgfb3 expression was unaltered. In addition, the expression of the TGF-β receptor Acvr1, also known as activin receptor-like kinase 1, was significantly enhanced in ankle joint synovium whereas Tgfr1, also known as activin receptor-like kinase 5, expression was similar between the two joints. Moreover, Axl and Gas6 expression were also significantly enhanced in ankle joint synovium (Fig. 4A). The expression of Tgfb1 and Axl in murine synovium significantly correlated (Fig. 4B). In human synovial RA biopsies, AXL and TGFB1 also showed a strong correlation, corroborating the murine data (Fig. 4C, Supplementary Fig. S4, available at Rheumatology online).

TGF-β1 potently induces AXL in human M0s, human synovial micromasses and murine knee joints

To further explore the effect of TGF-β1 on AXL in macrophages, M2-like M-CSF M0s were stimulated with TGF-β1. The expression of AXL and PLOD2, a gene regulated by TGF-β1 [28], were significantly enhanced by TGF-β1 (Fig. 5A). The TGF-β1-induced AXL expression on M2-like M-CSF M0s was confirmed on protein level by flow cytometry (Fig. 5B). In contrast, RAFLS did respond to

WT mice were injected intra-articularly into the knee joint with Ad Luc or Ad TGF-β1. (A–C) At day 3 and day 7, knee synovial biopsies were obtained. Messenger RNA was extracted and gene expression was determined. Data are represented as dot-plots with mean. n = 5 mice per group. * = P < 0.05 with Mann–Whitney test. (B–D) At day 3 and day 7, knee joints were processed for histology and sections were examined for AXL, F4/80 and CD206 protein expression. Shown are representative pictures of immunohistochemical synovial staining of AXL in 200× magnification. Staining is representative for six mice. Ad: Adenovirus; Luc: Luciferase; ND: not detected; B: bone; BM: bone marrow; S: synovium; WT: wild-type.
Discussion

This study shows that AXL plays a protective role in inflammatory arthritis in a spatial manner whereby it protects ankle joints but not knee joints. The distinct anatomical location of AXL expression appears to be regulated by TGF-β1. We show that TGF-β1-induced AXL expression is preserved in human sentinel cells and that AXL dampens their activation by controlling IL-1β secretion.

The symmetry and distinct anatomical locations of affected joints are clinically significant signs of RA [29]. However, the foundation for such selective positional manifestation of RA is an unsolved scientific knowledge gap. Our data show that the ratio of M2-like over M1-like macrophages is increased in ankle vs knee synovium. These findings are in line with the fact that ankle synovial CD64hi macrophages express lower levels of MHC II+ compared with the knee synovial macrophages [30]. In addition, we show a robust difference in the expression of the anti-inflammatory tyrosine kinase receptor AXL between ankle and knee synovium. Raghu and colleagues elegantly showed that plasminogen can be either deleterious or ameliorating in TNF-α-driven experimental arthritis, dependent on the anatomic location [31]. These data all corroborate the differences between spatially distinct joints.

We cannot exclude that there were no, or hardly any macrophages, in the synovium of the murine knee joints based on this study. However, it appears that the cells in the murine knee joints were not strongly positive for F4/80, a macrophage marker. However, there are also F4/80low macrophages [32, 33]. Moreover, some CD206+ cells, a marker for M2-like macrophages, appeared to be present in the murine knee joint synovium. Furthermore, we also cannot rule out that the correlation we find between TGFβ1 and AXL expression in synovial tissue is (also) due to other cells than macrophages. However, we can eliminate the fact that TGF-β1 can enhance AXL expression in synovial fibroblasts. The role of AXL is cell-type specific [34]. It has been described that endothelial cells and smooth muscle cells in synovial tissue can express AXL [17]. The endothelium also contains CD68+ macrophages that express AXL [35]. As O’Donnell and colleagues did not show co-localization of AXL with any other markers, we cannot exclude that these cells were macrophages. Moreover, a recent publication showed that the CD68+ synovial lining macrophages predominantly express AXL [36].

The articular cartilage in the ankle joint experiences the highest contact force of all the major joints [37–39]. Loading articular cartilage leads to the activation of TGF-β1, which in turn induces TGF-β1 in a positive feedback loop [40]. Therefore, the enhanced force experienced in the ankle, compared with the knee, could explain the increased levels of Tgfβ1 we observed in the ankle compared with the knee joint synovium. As TGF-β1 is a strong inducer of M2-like macrophage differentiation [24, 25], this could explain the dominance of these cells. Knee joints overexpressing TGF-β1 had more macrophages compared with the control virus. Moreover, these cells were also positive for the M2-like marker CD206. This shows that the murine knee joint was not intrinsically impaired in expressing AXL but does not express AXL under naïve conditions.

TGF-β1 but did not enhance AXL expression (Supplementary Fig. 5, available at Rheumatology online). Synovial micromasses, a model for the human synovial membrane containing synovial-like fibroblasts and CD68+ and CD163+ macrophages, which express AXL (Supplementary Fig. 6, available at Rheumatology online) when stimulated with TGF-β1, also expressed higher levels of AXL and PLOD2 (Fig. 5C). In M2-like M-CSF Mφs, TGF-β1 reduced MERTK and GAS6 expression, whereas in synovial micromasses, TGF-β1 did not alter their expression (Fig. 5A–C). Next, we adenosively overexpressed active TGF-β1 in murine knee joints, a tissue devoid of AXL during homeostasis (Fig. 2). Ad-TGF-β1 injected intra-articularly into the knee joint of naïve mice resulted in a significant increase of pTGFβ1 expression (Fig. 6A and C). As found in human Mφs and synovial micromasses, Axl was significantly upregulated at day 3 and day 7 after Ad-TGF-β1. In contrast, Gas6 expression was significantly upregulated at day 7 whereas Mertk gene expression was not differently expressed at either time point (Fig. 6A and C). The enhanced expression of Axl by Ad-TGF-β1 was confirmed on protein level (Fig. 6B and D). Knee joints overexpressing TGF-β1 had more F4/80+ macrophages compared with the control virus. Moreover, some of these cells were also positive for the M2-like marker CD206. This shows that AXL dampens their activation by controlling IL-1β secretion.

AXL expression is preserved in human sentinel cells and that AXL dampens their activation by controlling IL-1β secretion.
soluble AXL and GAS6 in RA patients were measured in plasma [19]. Furthermore, AXL expression was significantly reduced in CD11c+ dendritic cells from blood of RA patients [14]. We show that the expression of AXL is joint-specific. For this, the plasma levels of GAS6 or AXL might not be indicative for the local role of synovial AXL in anatomically distinct joints in RA patients.

To conclude, TGFβ1 and AXL expression significantly correlate in human and murine synovium. Local differences in AXL expression in mice lead to joint-specific differences in arthritis severity and the evidence provided suggest that these local differences in AXL might be due to TGF-β1. The TGF-β1-induced AXL expression and its anti-inflammatory properties are conserved in human macrophages and human synovial micromasses. These data suggest that AXL is a potential therapeutic target in RA, dependent on the joint(s) affected.

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Supplementary data
Supplementary data are available at Rheumatology online.

References


46 Fritz HK, Gustafsson A, Ljungberg B et al. The Axl-regulating tumor suppressor miR-34a is increased in ccRCC but does not correlate with Axl mRNA or Axl protein levels. PLoS one 2015;10:e0135991.