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Original article

Promotion of macrophage activation by Tie2 in the context of the inflamed synovia of rheumatoid arthritis and psoriatic arthritis patients

Pawel A. Kabala[1,2], Beatriz Malvar-Fernández[1,2], Ana P. Lopes[1,2], Tiago Carvalheiro[1,2], Sarita A. Y. Hartgring[1,2], Man Wai Tang[3,4], Carmen Conde[5], Dominique L. Baeten[3,4], Matthew Sleeman[6], Paul P. Tak[4,7,8], Jane Connor[9], Timothy R. Radstake[1,2,*], Kris A. Reedquist[1,2,*] and Samuel García[1,2,*]

Abstract

Objective. To examine the role of Tie2 signalling in macrophage activation within the context of the inflammatory synovial microenvironment present in patients with RA and PsA.

Methods. Clinical responses and macrophage function were examined in wild-type and Tie2-overexpressing (Tie2-TG) mice in the K/BxN serum transfer model of arthritis. Macrophages derived from peripheral blood monocytes from healthy donors, RA and PsA patients, and RA and PsA synovial tissue explants were stimulated with TNF (10 ng/ml), angiopoietin (Ang)-1 or Ang-2 (200 ng/ml), or incubated with an anti-Ang2 neutralizing antibody. mRNA and protein expression of inflammatory mediators was analysed by quantitative PCR, ELISA and Luminex.

Results. Tie2-TG mice displayed more clinically severe arthritis than wild-type mice, accompanied by enhanced joint expression of *IL6*, *IL12B*, *NOS2*, *CCL2* and *CXCL10*, and activation of bone marrow-derived macrophages in response to Ang-2 stimulation. Ang-1 and Ang-2 significantly enhanced TNF-induced expression of pro-inflammatory cytokines and chemokines in macrophages from healthy donors differentiated with RA and PsA SF and peripheral blood-derived macrophages from RA and PsA patients. Both Ang-1 and Ang-2 induced the production of IL-6, IL-12p40, IL-8 and CCL-3 in synovial tissue explants of RA and PsA patients, and Ang-2 neutralization suppressed the production of IL-6 and IL-8 in the synovial tissue of RA patients.

Conclusion. Tie2 signalling enhances TNF-dependent activation of macrophages within the context of ongoing synovial inflammation in RA and PsA, and neutralization of Tie2 ligands might be a promising therapeutic target in the treatment of these diseases.

Key words: rheumatoid arthritis, psoriatic arthritis, macrophages, Tie2, angiopoietins, inflammation, serum transfer-induced arthritis, synovial tissue explants

BASIC SCIENCE

Rheumatology key messages

- Tie2 overexpression enhances the severity of K/BxN serum transfer-induced arthritis.
- Tie2 induces the expression of inflammatory mediators by macrophages from RA and PsA patients.
- Ang-2 neutralization reduced the production of IL-6 and IL-8 by synovial tissue of RA patients.

[1]Department of Rheumatology and Clinical Immunology, [2]Laboratory of Translational Immunology, University Medical Center Utrecht, University of Utrecht, Utrecht, [3]Department of Experimental Immunology, [4]Department of Clinical Immunology and Rheumatology, University of Amsterdam, Amsterdam, The Netherlands, [5]Laboratorio de Investigación 8 y Servicio de Reumatología, Instituto de Investigación Sanitaria (IDIS), Hospital Clínico Universitario de Santiago de Compostela (CHUS), SERGAS, Santiago de Compostela, Spain, [6]Regeneron Pharmaceuticals, Inc., Tarrytown, NY, USA, [7]GlaxoSmithKline, Stevenage, [8]Department of Medicine, University of Cambridge, Cambridge, UK and [9]MedImmune LCC, MD, USA

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*Timothy R. Radstake, Kris A. Reedquist and Samuel García contributed equally to this study.

Correspondence to: Samuel García, Department of Rheumatology and Clinical Immunology, Laboratory of Translational Immunology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. E-mail: S.GarciaPerez@umcutrecht.nl

Introduction

RA and PsA are clinically distinct diseases with different aetiologies, but both are characterized by synovial hyperplasia, elevated synovial expression of proinflammatory cytokines including TNF, IL-1β and IL-6, increased angiogenesis and eventual joint destruction [1, 2]. Tie2 is a tyrosine kinase receptor essential for vascular development and blood vessel remodelling through interaction with its ligands angiopoietin-1 (Ang-1) and Ang-2 [3]. Tie2 and its ligands are expressed in RA and PsA synovial tissue at higher levels than observed in healthy controls and OA patients [4–6]. In RA and PsA synovial tissue, Tie2 is expressed by fibroblast like synoviocytes (FLS), endothelial cells (ECs) and macrophages [5, 7]. Ang-1 and Ang-2 are produced by synovial ECs, macrophages and FLS, and their expression is induced by inflammatory mediators such as TNF, TGF-β and toll-like receptors agonists [4–6, 8–10]. Importantly, expression of Tie2, Ang-1 and Ang-2 is elevated in the early stages of RA and PsA, and Tie2 signalling may contribute to the onset and progression of RA [6, 11]. Moreover, inhibition of Tie2 signalling using gene therapy strategies, soluble receptors or neutralizing antibodies against Ang-2 or Tie2 reduces the incidence and severity of murine experimental arthritis [7, 12–15].

We have previously observed that phosphorylated Tie2, indicative of Tie2 activation, is predominantly observed in RA and PsA synovial macrophages [7]. Macrophages are one of the most predominant cell types present in the synovium of RA and PsA patients and contribute to the pathology of these diseases through the release of inflammatory cytokines, MMPs, chemokines, reactive oxygen and nitrogen intermediates [16–18]. Importantly, clinical disease activity in RA and PsA correlates strongly with macrophage numbers in the synovial tissue and with the production of macrophage-derived cytokines like TNF, IL-1β and IL-6. Reciprocally, decreased numbers of synovial macrophages and expression of macrophages products correlate strongly with the clinical efficacy of treatments in RA and PsA [19–22]. Myeloid Tie2 signalling has been most extensively studied in tumour-associated Tie2-expressing monocytes, which have immunosuppressive properties and play an essential role in the vascularization and growth of solid tumours [23–26]. However, we found *in vitro* that Tie2 is expressed by both M1 (pro-inflammatory) and M2 (immunoregulatory) macrophages differentiated from healthy donors. While stimulation of these cells with Ang-1 or Ang-2 had little effect on macrophage gene expression, Tie2 stimulation of both M1 and M2 macrophages enhanced TNF-induced expression of cytokines and chemokines [27].

In vitro polarization of cells is a useful model for studying potential immunomodulatory properties of macrophages under different disease conditions, and synovial macrophages from inflammatory arthritis patients display both M1 and M2 characteristics [28]. However, the complex cytokine and cell–cell interactions present in the inflamed joint confer a unique pro-inflammatory transcriptome to synovial macrophages [29, 30] that can alter macrophage responses to subsequent cytokine stimulation through signalling crosstalk, positive and negative feedback mechanisms, and epigenetic regulation [31–33]. As a result, a cytokine signalling pathway that by itself has robust effects on macrophage gene expression and activation may make inconsequential or unpredictable contributions to synovial pathology within a complex inflammatory environment. Notably, although expression of Tie2 and its ligands is altered in both RA and PsA synovial tissue, differences in neo-angiogenic patterning is one of the most readily distinguishing features between these diseases, which are otherwise very similar in cellular composition and gene expression [6, 34, 35]. Given these considerations, we here examined how Tie2 stimulation might regulate macrophage activation in the context of the synovial environment, using murine and *ex vivo* human models of inflammatory arthritis.

Methods

Methodology describing the histological analysis, isolation of murine bone marrow, human monocytes, flow cytometry, RT-PCR and quantitative (q)PCR arrays, immunoblotting, measurement of cytokine production and the culture, differentiation and stimulation of macrophages and synovial biopsy explants has been previously published and is provided in detail in the Supplementary Material, section Methods, available at *Rheumatology* online [27, 36, 37].

Generation and maintenance of Tie2-overexpressing mice

Transgenic mice inducibly overexpressing Tie2 in all cellular compartments normally expressing endogenous Tie2 (Tie2-TG) were described previously [38]. pTek-tTA and pTetOS-Tek mice (kindly provided by D. Dumont, Sunnybrook Research Institute, Toronto, ON, Canada), maintained as CD1 outbred lines, were backcrossed onto a wild-type (WT) C57BL/6J background for at least 10 generations prior to their use in experiments. Tie2-TG and WT control littermates were obtained by breeding the pTek-tTA and the pTetOS-Tek mouse lines and identification by genotyping. Mice were housed under conventional conditions at the animal facility of the Academic Medical Center (Amsterdam, The Netherlands) and were fed *ad libitum*. The drinking water of mice was supplemented with doxycycline (200 μg/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands) until 2 weeks before initiation of experiments, at which time doxycycline was withdrawn to allow overexpression of Tie2. Animal experiments were approved by the Academic Medical Center animal ethics committee.

Serum-transfer arthritis and clinical scoring

K/BxN serum was collected from 4- to 8-week-old arthritic K/BxN mice (provided by C. Benoist and D. Mathis, Harvard Medical School, Boston, MA, USA), pooled and stored at −80°C until use. Arthritis was induced by transfer of 100 μl of K/BxN serum into 12- to 14-week-old mice (five WT and five Tie2-TG) by intraperitoneal injection on day 0 and day 2. Mice were sacrificed on day 14 after

serum transfer. Arthritis severity was assessed in each of the four limbs, every 2 days by two blinded observers, using a semi-quantitative clinical score (0: no swelling; 1: slight swelling and erythema of the ankle, wrist or digits; 2: moderate swelling and erythema; 3: severe swelling and erythema; and 4: maximal inflammation with joint rigidity; maximum possible score 16 per mouse).

Patients

Synovial biopsies were obtained by needle arthroscopy as previously described [39] from clinically active inflamed joints of RA and PsA patients fulfilling the ACR/EULAR criteria for RA and the classification criteria for PsA, respectively [40–42]. Clinical characteristics of patients providing synovial tissue, peripheral blood, serum and SF for cellular material are detailed in supplementary Tables S1–S3, available at *Rheumatology* online. All patients provided written informed consent prior to their inclusion in this study, and this study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam (MEC 07/253) and the University Medical Center Utrecht (MEC 13-696 and 13-697).

Statistical analyses

Statistical analysis was performed using Windows GraphPad Prism 5 (GraphPad Software, Inc.). Potential differences between experimental groups were analysed by non-parametric, Kruskal–Wallis test and Friedman test, or parametric analysis of variance test as appropriate. *P*-values <0.05 were considered statistically significant.

Results

Increased arthritis severity in mice overexpressing Tie2

Interfering with Tie2 signalling by administration of soluble recombinant Tie2 or neutralizing anti-Ang-2 antibodies is protective in murine models of arthritis [7, 13], but it is unclear whether the overexpression of Tie2 observed in the synovium of RA patients is sufficient to enhance inflammatory pathways *in vivo* [4–6]. Therefore, we determined the consequences of altered Tie2 expression on disease activity in the K/BxN serum transfer murine arthritis model, using mice in which removal of deoxycycline from the drinking water allows overexpression of transgenic Tie2 in all cell types expressing endogenous Tie2 [38]. The administration of K/BxN serum led to arthritis in all of the WT and Tie2-TG mice (data not shown). The clinical severity of disease was significantly higher in Tie2-TG mice (Fig. 1A). Importantly, we observed an overexpression of Tie2 in the total joint tissue of Tie2-TG arthritic mice, as well as in isolated bone marrow-derived macrophages (BMDM, Fig. 1B–D and supplementary Fig. S1A, available at *Rheumatology* online). Histological analysis of the tibiotalar and forefoot joints revealed a significant increase in synovial inflammation in Tie2-TG mice compared with WT mice, and a trend towards increased bone erosion. Tie2 overexpression did not influence cartilage damage in this arthritis model (Fig. 1E and F). To determine the molecular mechanisms associated with the more severe clinical pathology observed in Tie2-TG mice, we analysed joint mRNA expression of inflammatory mediators, either identified previously as Tie2-inducible gene products in human macrophages or well-documented contributors to pathology in murine arthritis [27]. In WT mice, the induction of arthritis up-regulated expression of *Il12b*, *Tnf* and *Ccl3*, compared with non-arthritic WT mice. In Tie2-TG arthritic mice, expression of *Il6*, *Il12*, *Nos*, *Il1*, *C-C motif ligand 2 Ccl2*, C-X-C motif chemokine *Cxcl10*, *Tnf*, *Ccl3*, *Mmp3* and *Tnfrsf11a* (the gene that encodes Receptor activator of nuclear factor $\kappa\beta$) was enhanced compared with non-arthritic WT and Tie2-TG mice. Comparing expression between arthritic WT and Tie2-TG mice, we observed that expression of *Il6*, *Il12B*, *Nos*, *Ccl2* and *Cxcl10* was significantly higher in Tie2-TG mice (Fig. 2A). We also analysed the expression of the Tie2 ligands. The expression of *Ang1* and *Ang2* was similar between the non-arthritic WT and Tie2-TG mice. Arthritis did not modulate the expression of *Ang1* and induced a slight up-regulation of *Ang2* in both WT and Tie2-TG mice arthritic mice, but differences were not significant (supplementary Fig. S1B, available at *Rheumatology* online).

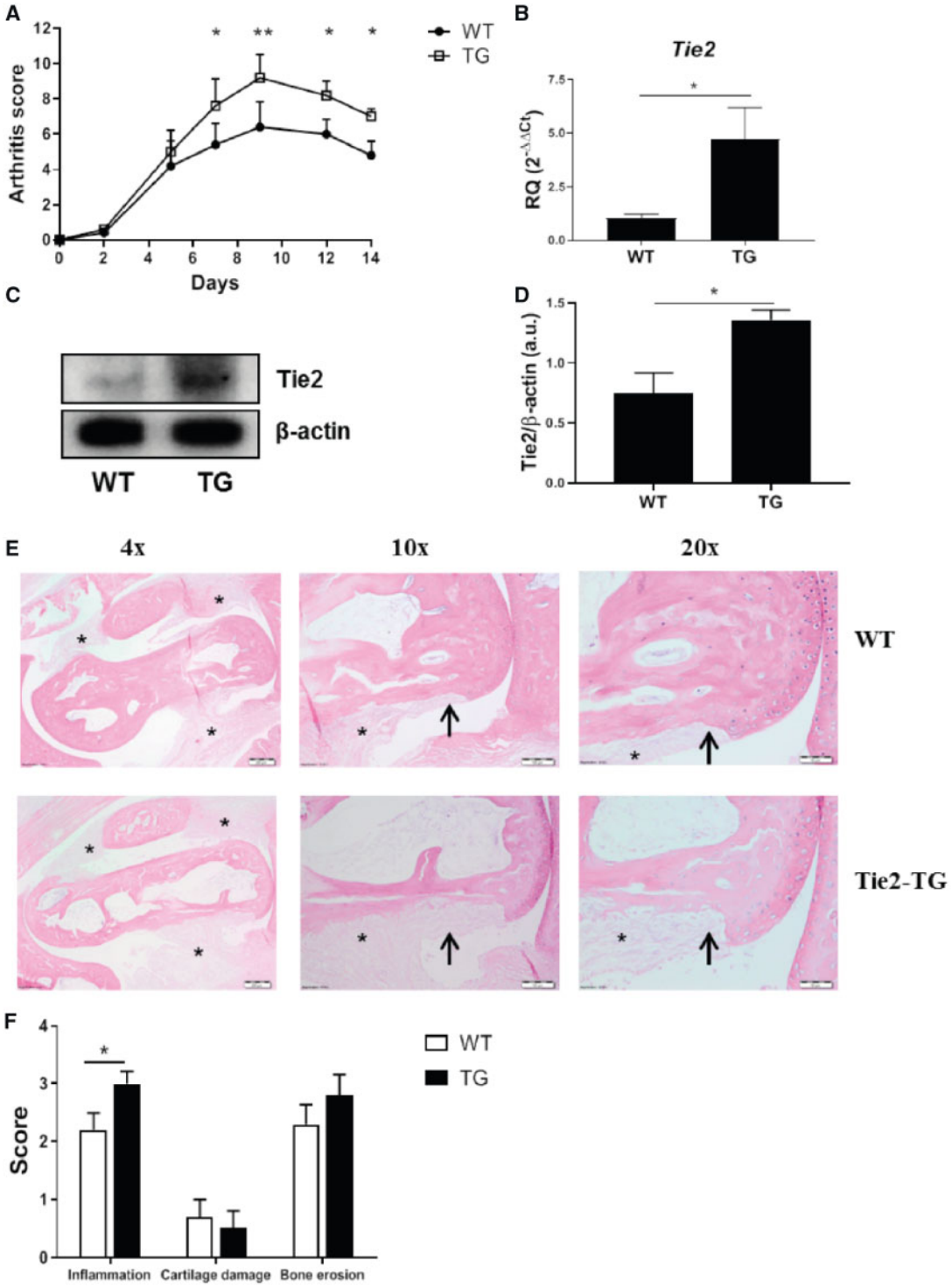
To determine whether Tie2 overexpression in murine macrophages could contribute to the enhanced pathology observed in arthritic Tie2-TG mice, we analysed the mRNA expression of these inflammatory mediators by unpolarized BMDM from untreated and arthritic WT and Tie2-TG mice. The expression of *Il6*, *Il12b*, *Tnf* and *Ccl3*, genes displaying enhanced expression in Tie2-TG joints, was significantly higher in BMDM from arthritic Tie2-TG compared with arthritic WT mice (Fig. 2B). We also examined the capacity of Ang-1 and Ang-2 to stimulate M1-differentiated BMDM. Basal production of IL-6, but not IL-12p40, was significantly higher in macrophages from Tie2-TG arthritic mice, compared with BMDM from healthy WT and Tie2-TG mice (Fig. 2C). Stimulating BMDM with Ang-1 had no significant effect on IL-6 or IL-12p40 secretion. However, Ang-2 induced a significant increase in IL-6 secretion in each of the four groups of macrophages analysed, and IL-6 production was higher in arthritic Tie2-TG macrophages than the other macrophage groups. Ang-2 also induced a significant increase of IL-12p40 secretion in WT arthritic, and in Tie2-TG control and arthritic mice. The production of IL-12p40 induced by Ang-2 in the arthritic Tie2-TG macrophages was higher compared with the other macrophage groups, although significant differences were reached only in comparison with healthy WT mice (Fig. 2C).

Therefore, overexpression of Tie2 leads to a more severe clinical phenotype in this arthritis model, marked by enhanced synovial expression of inflammatory mediators produced by macrophages in response to Tie2 signalling.

The synovial microenvironment of RA and PsA supports functional expression of macrophage Tie2

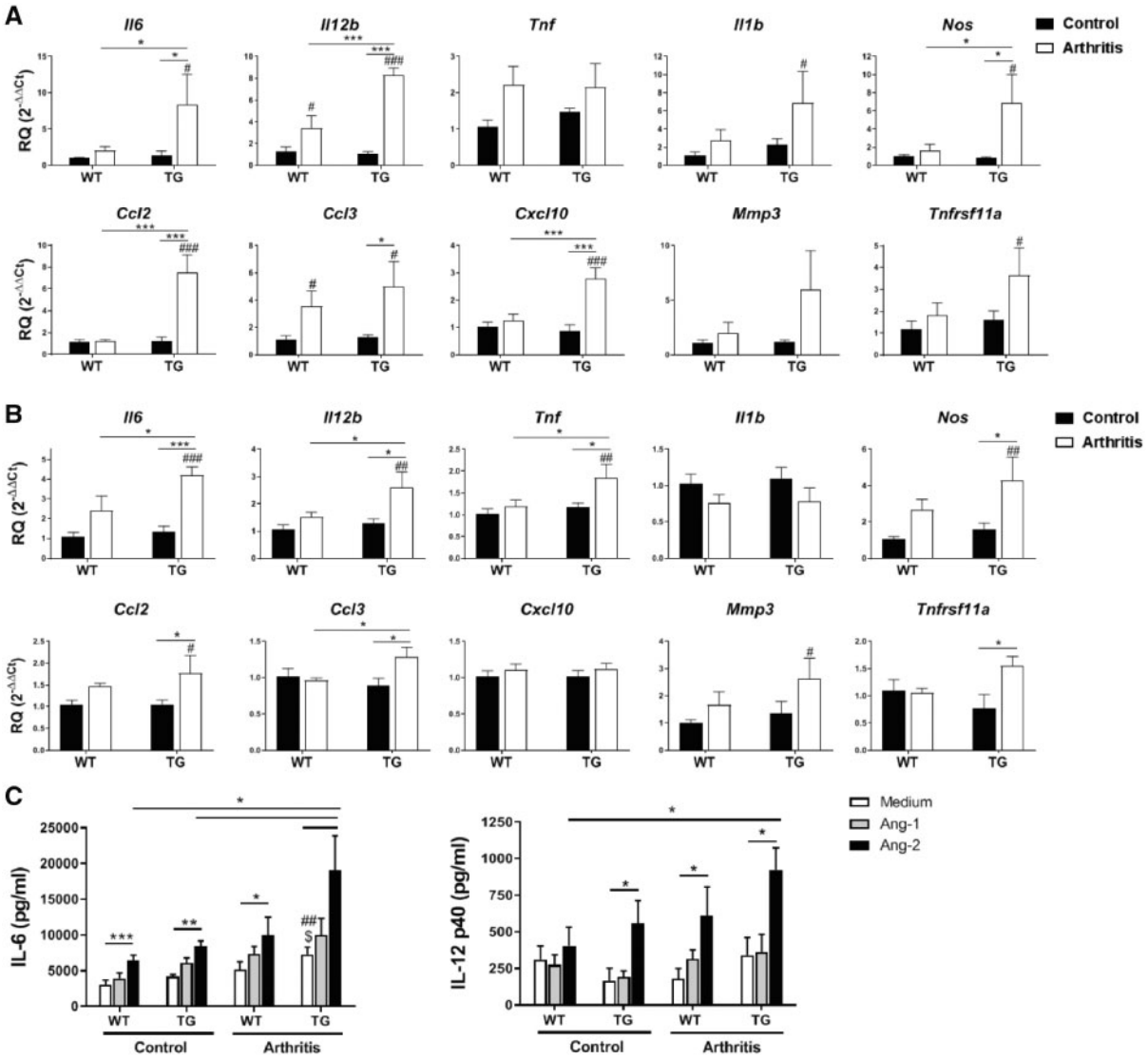
As Tie2 is activated in RA synovial macrophages [7], we determined how secreted products present in the synovial

Fig. 1 Increased arthritis severity in mice overexpressing Tie2



(**A**) Daily global arthritic scores of WT and Tie2-TG mice. *$P < 0.05$ and **$P < 0.01$ compared with WT mice. (**B, C**) mRNA expression (B) and representative immunoblot (C) of Tie2 in the joints of WT and Tie2-TG mice. qPCR data are shown as relative quantity respect to WT control mice, as described in the Methods. Bars represent the means and S.E.M. of five mice. *$P < 0.05$. (**D**) Densitometric analysis of Tie2 protein expression. Data are shown as relative expression with respect to β-actin expression. Bars represent the mean and S.E.M. of five mice. *$P < 0.05$. (**E**) Representative images of joint pathology in indicated mice visualized by haematoxylin and eosin staining. Representative areas of synovial cellular infiltration (asterisk) and bone erosion (black arrows) are noted. (**F**) Inflammation, cartilage damage and bone erosion scores of mice in each group. Data are mean ± S.E.M. for each group ($n = 10$ ankle joints per group). *$P < 0.05$. qPCR: quantitative PCR; Tie2-TG: transgenic mice inducibly overexpressing Tie2; WT: wild-type.

FIG. 2 Enhanced expression of inflammatory mediators in arthritic mice overexpressing Tie2

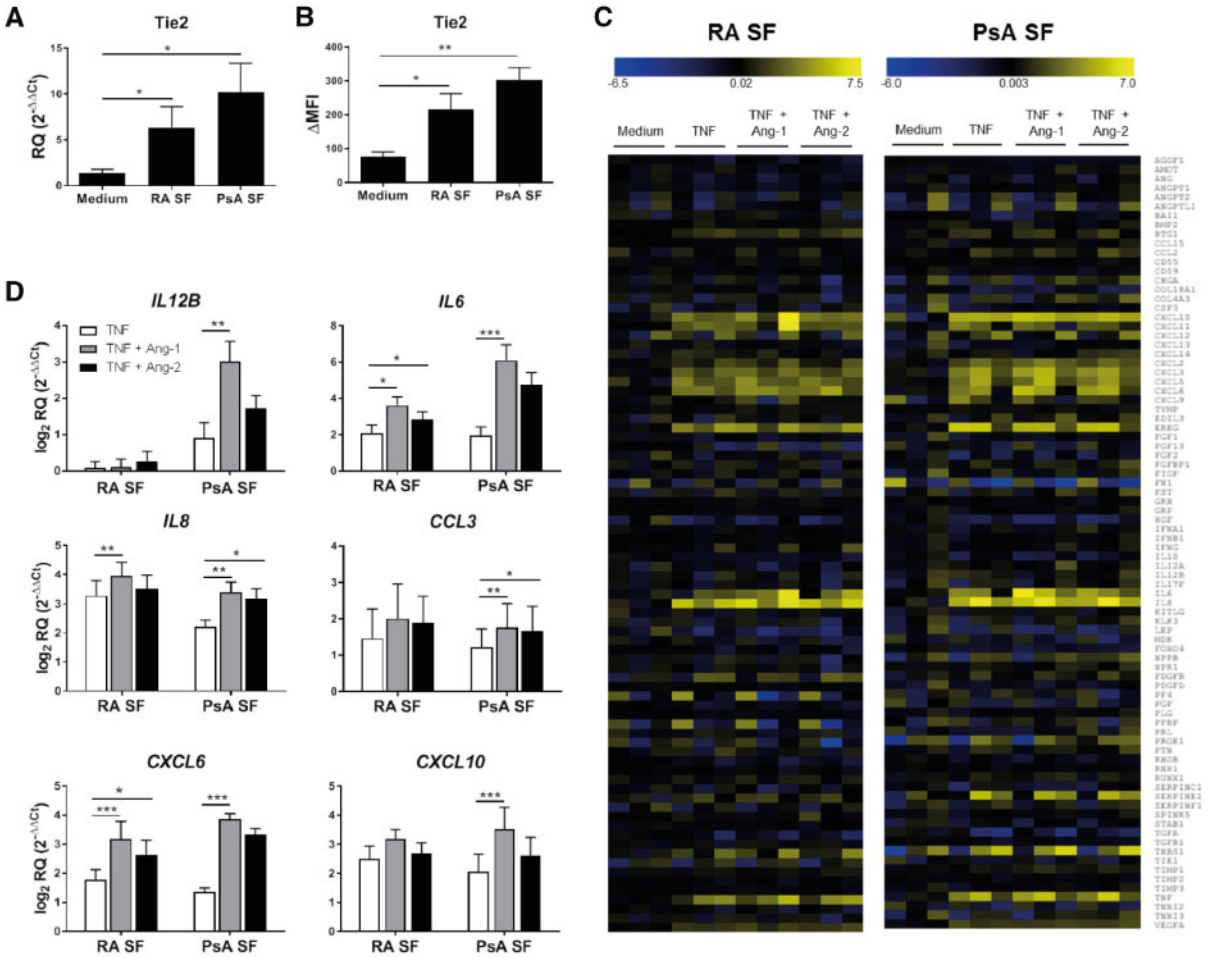


(**A, B**) mRNA expression of inflammatory mediators in the forepaws (A) and BMDM (B) of control and arthritic WT or Tie2-TG mice. Data are shown as relative quantity with respect to WT control mice, as described in the Methods. Bars represent the means and S.E.M. of five mice. (**C**) IL-6 and IL-12p40 expression in M1-differentiated BMDM from control and arthritic WT and Tie2-TG mice after 24 h incubation in medium, Ang-1 or Ang-2 (both 200 ng/ml). Bars represent the means and S.E.M. of five mice. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with WT control mice; $^{\$}P < 0.05$ compared with TG control mice. Ang-1 and -2: angiopoietin-1 and -2; BMDM: bone marrow-derived macrophages; Tie2-TG: transgenic mice inducibly overexpressing Tie2; WT: wild-type.

microenvironment might influence macrophage Tie2 expression and function. In one line of analysis, monocytes from peripheral blood (PB) of healthy donors (HD) were differentiated into macrophages with SF from RA ($M\Phi_{RA\ SF}$) and PsA ($M\Phi_{PsA\ SF}$) patients. Both RA and PsA SF induced significantly higher Tie2 mRNA (Fig. 3A) and protein (Fig. 3B) expression compared with macrophages differentiated with medium alone. However, serum of RA or PsA patients did not induce the expression of Tie2, suggesting that this effect is dependent upon the synovial microenvironment (supplementary Fig. S2, available at *Rheumatology* online).

In $M\Phi_{RA\ SF}$, Tie2 stimulation with Ang-1 or Ang-2 alone did not induce any significant change in mRNA expression of 84 angiogenesis-related genes (data not shown), negative results comparable to those previously observed examining M1 and M2 macrophages obtained from HD, where Tie2 signalling only influenced macrophage gene expression in combination with TNF stimulation [27]. We therefore stimulated $M\Phi_{RA\ SF}$ and

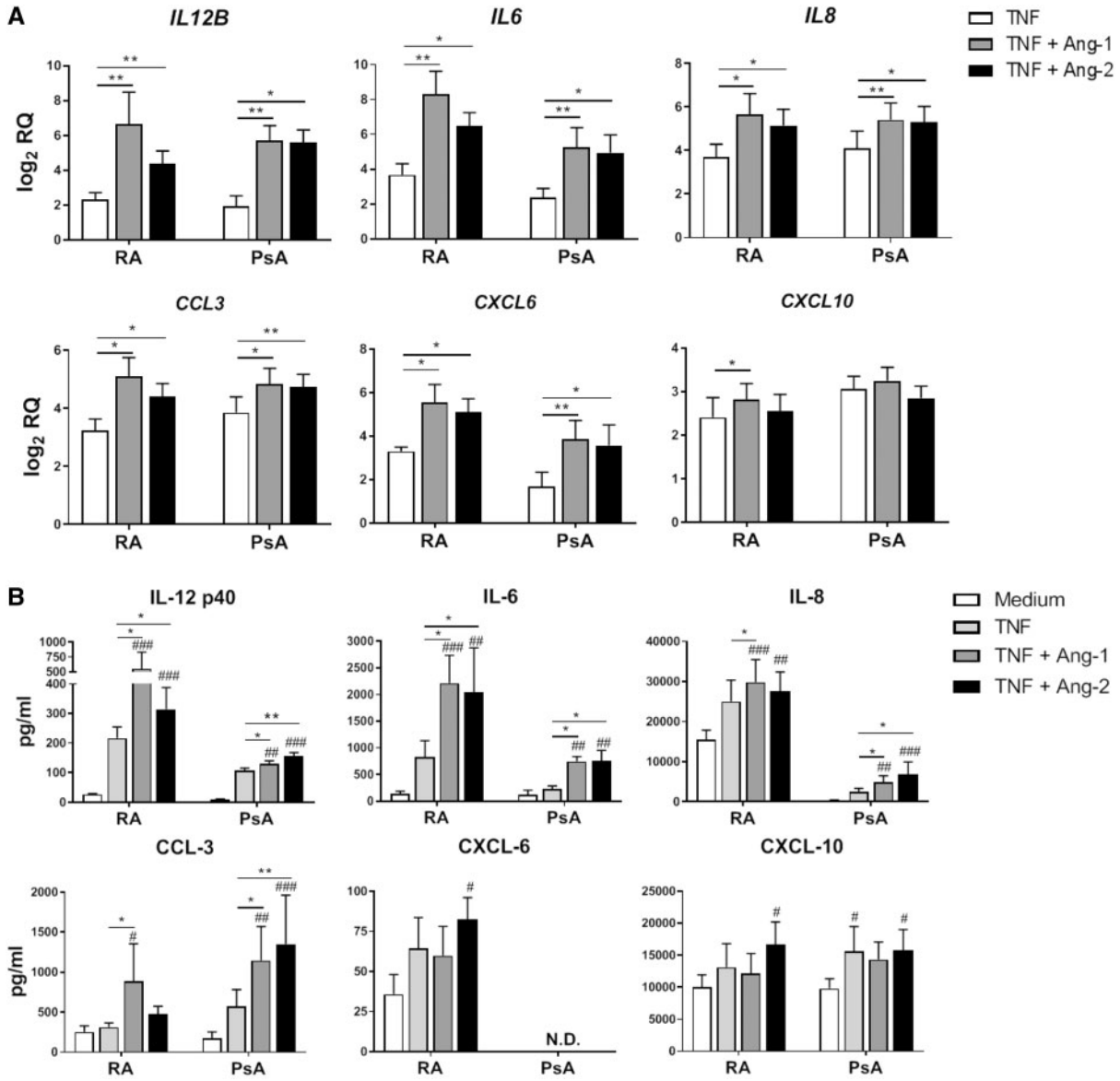
FIG. 3 Tie2 is expressed by macrophages differentiated with SF of RA or PsA patients and enhances the TNF-induced mRNA expression of cytokines and chemokines



(**A, B**) mRNA (A) and protein (B) Tie2 expression in macrophages differentiated with medium, or SF of RA or PsA patients for 7 days. qPCR data are shown as relative quantity, as described in the Methods. Surface expression was calculated as ΔMFI = (Median fluorescence intensity)$_{\text{positive staining}}$ − (Median fluorescence intensity)$_{\text{isotype staining}}$. Values are the mean ± S.E.M. of seven independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. (**C**) mRNA expression profiles of angiogenesis related genes in macrophages differentiated with SF of RA or PsA patients after 4 h incubation in medium alone or TNF (10 ng/ml) in the absence or presence of Ang-1 or Ang-2 (200 ng/ml for both, $n = 3$). Data are presented as a heat map where the lowest mRNA expression is showed in dark blue and the highest in yellow. (**D**) Validation of mRNA expression levels of selected genes analysed in (C). Data are shown as $\log_2$ relative quantity respect to unstimulated cells, as described in the Methods. Bars represent the means and S.E.M. of six independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Ang-1 and -2: angiopoietin-1 and -2; qPCR: quantitative PCR.

$M\Phi_{PsA\ SF}$ with TNF in the absence or presence of Ang-1 or Ang-2. TNF stimulation up-regulated 13 genes at least 2-fold in $M\Phi_{RA\ SF}$. Of these genes, Ang-1 and Ang-2 enhanced the TNF-induced expression of six and five genes, respectively (Fig. 3C and supplementary Table S6, available at *Rheumatology* online). In $M\Phi_{PsA\ SF}$, TNF up-regulated 15 genes, of which Ang-1 and Ang-2 further enhanced the expression of 5 and 4 genes, respectively. To validate the array data, we analysed expression of *IL12B*, *IL6*, *CXCL6*, *CXCL8*, *CXCL10* and *CCL3* by qPCR. In $M\Phi_{RA\ SF}$, Ang-1 significantly enhanced TNF-induced expression of *IL6*, *IL8* and *CXCL6* (Fig. 3D), while Ang-2 similarly enhanced expression of *IL6* and *CXCL6*. In $M\Phi_{PsA\ SF}$ Ang-1 significantly enhanced TNF-induced expression of *IL12B*, *IL6*, *IL8*, *CCL3*, *CXCL6* and *CXCL10*, while Ang-2 enhanced TNF-induced expression of *IL8* and *CCL3* (Fig. 3D). These results suggest that unidentified secreted factors present in the synovial microenvironment of RA and PsA patients can induce the expression of functional Tie2, which in turn can participate in the pro-inflammatory activation of macrophages.

FIG. 4 Ang-1 and Ang-2 enhance the TNF-induced mRNA and protein expression of inflammatory mediators in RA and PsA macrophages



(**A**) mRNA expression of chemokines and cytokines by monocytes from PB of RA and PsA patients differentiated with IFN-γ after 4 h incubation in medium alone or TNF (10 ng/ml) in the absence or presence of Ang-1 or Ang-2 (200 ng/ml for both). Data are shown as $\log_2$ relative quantity with respect to unstimulated cells, as described in the Methods. Bars represent the means and S.E.M. of five independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$. (**B**) Analysis of protein production by monocytes from PB of RA and PsA patients differentiated with IFN-γ after 24 h incubation in medium alone or TNF (10 ng/ml) in the absence or presence of Ang-1 or Ang-2 (200 ng/ml for both). Bars represent the means and S.E.M. of five independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with unstimulated cells. Ang-1 and -2: angiopoietin-1 and -2; PB: peripheral blood.

Tie2 signalling promotes the pro-inflammatory activation of PB-derived RA and PsA macrophages

To determine whether systemic inflammation could also impact upon macrophage Tie2 expression and function, we obtained PB monocytes from HD and RA and PsA patients, and differentiated them into macrophages using either IFN-γ ($M\Phi_{IFN\text{-}\gamma}$) or IL-10 ($M\Phi_{IL\text{-}10}$), as macrophages present in RA and PsA synovial tissue are

phenotypically similar to macrophages differentiated with these cytokines [27, 28]. Surface expression of Tie2 was similar in $M\Phi_{IFN\text{-}\gamma}$ and $M\Phi_{IL\text{-}10}$ derived from HD and patients, and no differences were observed in Tie2 expression between macrophages derived from PB and SF of RA patients (supplementary Fig. S3A and B, available at *Rheumatology* online). In $M\Phi_{IFN\text{-}\gamma}$, both Ang-1 and Ang-2 significantly enhanced TNF-induced expression of *IL12B*, *IL6*, *IL8*, *CXCL6* and *CCL3* by RA and PsA macrophages (Fig. 4A), and Ang-1 also increased *CXCL10* expression in RA patient $M\Phi_{IFN\text{-}\gamma}$. This pro-inflammatory effect of Tie2 stimulation was not unique to $M\Phi_{IFN\text{-}\gamma}$, as Tie2 stimulation also enhanced expression of inflammatory mediators in $M\Phi_{IL\text{-}10}$ from RA and PsA patients (supplementary Fig. S4, available at *Rheumatology* online). We also analysed the protein expression of these inflammatory mediators in tissue culture supernatants of stimulated $M\Phi_{IFN\text{-}\gamma}$ (Fig. 4B). In $M\Phi_{IFN\text{-}\gamma}$ differentiated from RA and PsA patients, Ang-1 and Ang-2 significantly enhanced TNF-induced secretion of IL-12p40, IL-6, IL-8 and CCL-3, but not CXCL-6 or CXCL-10. To determine whether other inflammatory cytokines involved in RA and PsA pathogenesis also cooperate with Tie2 signalling in the activation of macrophages, we stimulated $M\Phi_{IFN\text{-}\gamma}$ from RA and PsA patients with IL-1β and IL-6 in combination with Ang-1 or Ang-2. Ang-1, and to a lesser extent Ang-2, enhanced the IL-1β-induced expression of *IL12B*, *IL6* and *IL8*. IL-6 slightly up-regulated the mRNA expression of these inflammatory mediators, but despite this modest effect, Ang-1 enhanced the expression of *IL6* and *IL8.* Ang-2 also enhanced the IL-6-induced expression of *IL6* and *IL8*, but the effect was less prominent (supplementary Fig. S5A and B, available at *Rheumatology* online). These results suggest a generalized pro-inflammatory role for Tie2 signalling within the context of other cytokines present in the synovium of RA and PsA patients. Finally, to understand how exposure of monocytes to the synovial microenvironment might influence Tie2 signalling, we differentiated RA patient SF monocytes with IFN-γ. Ang-1 and Ang-2 significantly enhanced TNF-induced expression of *IL12B*, *IL6*, *IL8*, *CXCL6* and *CCL3*, but not *CXCL10* (supplementary Fig. S6A, available at *Rheumatology* online). Enhanced Tie2-dependent production of IL-6 and IL-8 was also confirmed at the protein level (supplementary Fig. S6B, available at *Rheumatology* online). These results confirm that the pro-inflammatory effect of Tie2 signalling in macrophages is preserved in cells exposed to systemic alterations in cytokine levels and the synovial microenvironment present in patients with inflammatory arthritis.

Tie2 signalling is sufficient and required for perpetuation of inflammation in RA synovial tissue

Lastly, we examined whether the pro-inflammatory effects of Tie2 signalling in isolated macrophages under conditions relevant to RA were preserved in the intact synovium of RA patients. Synovial tissue explants from RA patients were incubated in medium alone, or in the presence of Ang-1 or Ang-2, and secreted analytes were measured. We observed that Ang-2 alone significantly induced the secretion of IL-12p40, IL-6, IL-8 and CCL-3 by RA synovial explants (Fig. 5A). In contrast, Ang-1 only significantly induced the secretion of IL-12p40 and CCL-3. TNF induced the secretion of CCL-3, but had a small or negligible effect on the production IL-6, IL-12p40 and IL-8. In contrast, the combination of TNF with Ang-1 or Ang-2 significantly induced the production of IL-6, IL-12p40, IL-8 and CCL-3, compared with the unstimulated explants (Fig. 5B). Interestingly, the pro-inflammatory effect of Ang-1 and Ang-2 was not restricted to RA, as Ang-2 significantly induced the secretion of IL-6 in the synovial tissue explants of PsA patients (supplementary Fig. S7, available at *Rheumatology* online).

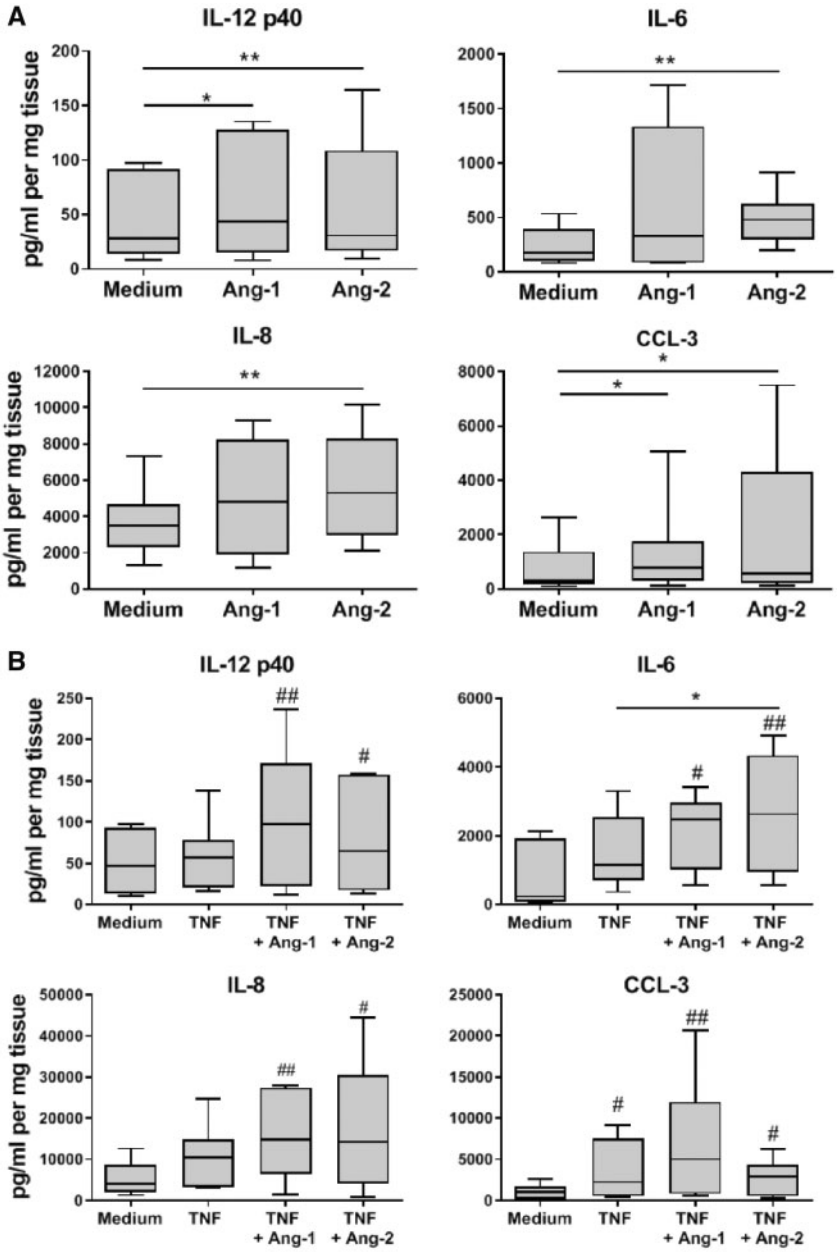
As Ang-2 appeared to regulate RA synovial tissue gene expression more robustly than Ang-1, we analysed the potential effects of blocking Ang-2 signalling in RA synovial explants using a neutralizing anti-Ang-2 antibody, which dose-dependently reduced *IL6* and *IL12B* expression in HD $M\Phi_{IFN\text{-}\gamma}$ macrophages stimulated with TNF and Ang-2, but not those stimulated with TNF and Ang-1 (Fig. 6A). In the RA synovial tissue explant model, Ang-2 neutralization significantly reduced IL-6 and IL-8 production compared with explants that were left untreated or exposed to isotype control antibody (Fig. 6B), providing direct evidence that Tie2 signalling induces macrophage expression of inflammatory mediators in the synovium of arthritis patients, and that specific neutralization of Ang-2 can reduce ongoing synovial expression of pro-inflammatory mediators relevant to disease in RA and other forms of arthritis.

Discussion

Expression of the Tie2 receptor tyrosine kinase and its ligands Ang-1 and Ang-2 is elevated in the synovium of RA and PsA patients compared with healthy and disease controls [7, 12, 13]. Intriguingly, expression and activation of Tie2 is elevated in the synovial tissue of early arthritis patients, associated with the eventual development of RA and disease progression [6, 11]. The potential importance of this to pathology in inflammatory arthritis is underscored by studies showing that inhibition of Tie2 signalling is protective in animal models of arthritis [7, 13–15]. Potential cellular targets of Tie2 signalling in the inflamed joint include ECs, FLS and macrophages. For example, toll-like receptor 2 and VEGF-mediated EC survival, migration and angiogenic tube formation is dependent upon Tie2 signalling [10, 43]. Tie2 stimulation of RA FLS promotes the chemotactic and tissue invasiveness of these cells, and TNF-dependent activation of macrophage chemokine and cytokine production is enhanced by Ang-1 and Ang-2 [7, 27, 44, 45]. An emerging unifying feature, from published observations and data presented here, is that Tie2 signalling cooperates with TNF and other agonists to promote cellular activation in inflammatory arthritis [7, 27, 46].

That Tie2 signalling exerts its effects predominantly within the context of cellular exposure to other cytokines, such as TNF and VEGF, is important for understanding

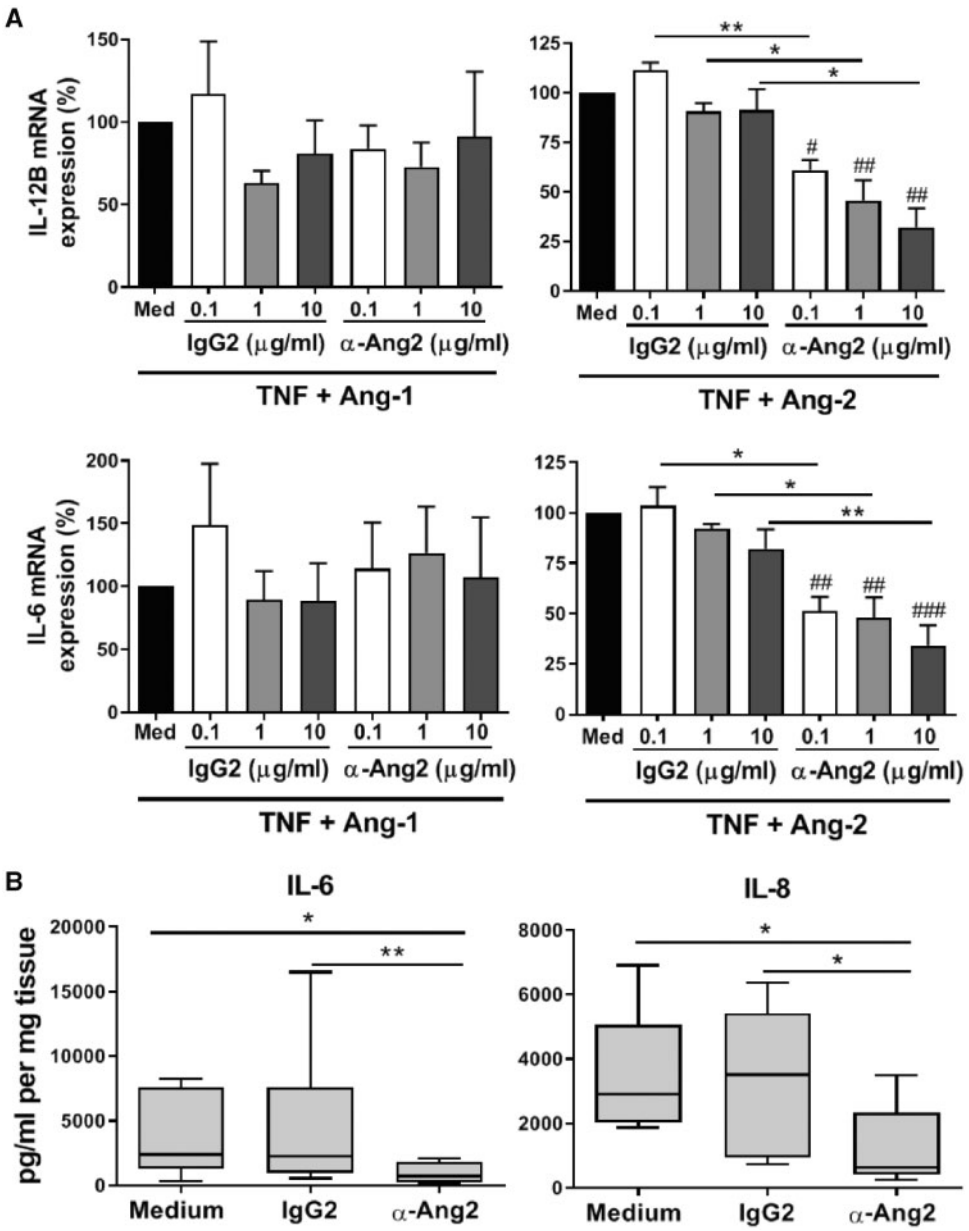
Fig. 5 Tie2 signalling induces cytokine and chemokine production in RA synovial tissue



(**A**) IL-6, IL-12p40, IL-8 and CCL-3 production in supernatants of RA synovial tissue after 24 h incubation in medium, Ang-1 or Ang-2 (both 200 ng/ml). (**B**) IL-6, IL-12p40, IL-8 and CCL-3 production in supernatants of RA synovial tissue after 24 h incubation in medium alone or TNF (10 ng/ml) alone or in combination with Ang-1 or Ang-2 (both 200 ng/ml). Data are presented as box plots, where the boxes represent the 25th–75th percentiles, the lines within the box mark the median value and lines outside the boxes denote the 10th and 90th percentiles ($n = 4$–7). $^{*}P < 0.05$ and $^{**}P < 0.01$; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with medium. Ang-1 and -2: angiopoietin-1 and -2.

and potentially targeting Tie2 contributions to pathology in inflammatory arthritis. First, macrophage responses to a given agonist are greatly influenced by the polarization conditions they are exposed to as they enter tissue. While synovial macrophages clearly make pro-inflammatory contributions in RA and PsA, their expression of both M1 (pro-inflammatory) and M2 (immunoregulatory) surface markers is consistent with exposure to IFN-γ and IL-10, respectively [47]. Global gene expression analysis of RA SF and synovial tissue macrophages confirms that the complex synovial microenvironment provides unique differentiation signals [30]. Second, responses of

FIG. 6 Ang-2 neutralization reduces cytokine production in RA synovial tissue



(**A**) mRNA expression of *IL6* and *IL12B* in RA synovial tissue explants stimulated for 24 h with TNF (10 ng/ml) plus Ang-1 (left panels) or Ang-2 (right panels, both 200 ng/ml) in the absence (med) or presence of control IgG2 or anti-Ang-2 antibodies. Data are presented as percentage of expression relative to TNF in combination with Ang-1 or Ang-2. Bars represent the means and S.E.M. of seven independent experiments. (**B**) IL-6 and IL-8 production in supernatants of RA synovial tissue after 24 h incubation in medium alone, anti-Ang2 or IgG2 antibody (0.1 μg/ml). Data are presented as box plots, where the boxes represent the 25th–75th percentiles, the lines within the box mark the median value and lines outside the boxes denote the 10th and 90th percentiles ($n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.01$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with medium. Ang-1 and -2: angiopoietin-1 and -2.

differentiated macrophage to a given cytokine are directly influenced by concurrent exposure to other cytokines and environmental influences. For example, IFN-γ, immune complexes and hypoxic conditions present in the inflamed synovium render macrophages refractory to immunoregulatory signalling by IL-10 [48, 49]. In contrast, TNF can tolerize macrophages to toll-like receptor 4 signalling while sensitizing them to type I IFN signalling [33].

Thus, it might not be possible to predict the contributions of macrophage Tie2 signalling to inflammatory arthritis using highly defined classical macrophage polarization models. To address this, we undertook three distinct experimental approaches, utilizing *in vivo*, *in vitro* and *ex vivo* models of RA. *In vivo*, Tie2 overexpression increased the clinical severity of murine serum-induced arthritis, consistent with previous data suggesting that elevated expression of angiopoietins and Tie2 in RA and PsA synovial tissue contributes to pathology [6, 11]. Enhanced disease activity in Tie2-TG mice was associated with the elevated expression of *IL6*, *IL12B*, *CCL-2* and *CXCL10*, inflammatory mediators that we had previously identified as inducible by Tie2 signalling in human macrophages [27]. BMDM from Tie2-TG arthritic mice also produced higher amounts of IL-6 than WT BMDM. These findings suggest that macrophage Tie2 signalling can contribute directly to inflammation in experimental arthritis. We did not address direct effects of enhanced Tie2 signalling in EC or FLS in these *in vivo* studies, questions that warrant further investigation.

In vitro, we examined the potential influences of systemic and synovial environmental cues on myeloid Tie2 expression and function in RA and PsA. PB monocytes from RA and PsA patients retained the capacity to upregulate expression of Tie2 following differentiation in either IFN-γ or IL-10. Tie2 expressed by these macrophages was functional, as Ang-1 and Ang-2 stimulation enhanced the production of cytokines and chemokines induced by different inflammatory mediators (TNF, IL-1β and IL-6), including IL-6, IL-8, IL-12p40 and CCL-3. We observed little if any difference in Tie2 expression between RA and PsA PB-derived macrophages, and only a slight trend towards increased sensitivity to Ang-2 in PsA, indicating that systemic disease influences did not overtly impact on myeloid Tie2 signalling. However, differentiation of HD PB monocytes in RA and PsA SF significantly up-regulated macrophage Tie2 mRNA and protein expression. Ang-1 and Ang-2 stimulation of these cells enhanced TNF-dependent expression of a subset cytokines and chemokines similar to what we previously observed in polarized HD macrophages [27]. Finally, we confirmed that macrophages derived from RA SF also retained the capacity to produce more TNF-dependent IL-6 and IL-8 in the presence of Ang-1 or Ang-2. Together, these results provide evidence that the synovial cytokine environment promotes myeloid Tie2 expression, and that Tie2 can cooperate with inflammatory mediators involved in the pathology of RA and PsA to promote macrophage cytokine and chemokine production in both RA and PsA. In the various models used in these studies, some quantitative but inconsistent differences were observed in Tie2-dependent gene expression between RA and PsA. No clear qualitative differences that might explain distinct neo-angiogenic patterning and myofibroblast gene signatures were observed [6, 34], possibly a limitation of the power of this study, and global gene expression analysis in these macrophages may yet reveal disease-specific differences in macrophage Tie2 signalling.

Lastly, we sought to determine whether Tie2 signalling was sufficient and necessary to sustain inflammatory gene expression in intact synovial tissue from patients with inflammatory arthritis *ex vivo*. Ang-2 alone induced RA synovial explant production of IL-6, IL-8, IL-12p40 and CCL-3, while Ang-1 enhanced only the production of IL-12p40 and CCL-3. In PsA synovial tissue, both Ang-1 and Ang-2 promoted IL-6 production. These results were in contrast to those obtained from isolated macrophages, where Tie2 signalling by itself did not affect gene expression, but likely due to the presence of inflammatory mediators such as TNF in patient synovial tissue [50]. Combinatorial exposure of RA synovial membrane to TNF, which by itself had little effect on cytokine and chemokine production, with Ang-1 or Ang-2 significantly enhanced production of IL-6, IL-12p40, IL-8 and CCL-3. Reciprocally, RA synovial tissue production of IL-6 and IL-8 was suppressed in the presence of a human neutralizing anti-Ang-2 antibody.

This study provides evidence that myeloid Tie2 signalling is preserved in RA and PsA synovial tissue, and engagement of Ang-2/Tie2 signalling is sufficient and necessary to promote synovial inflammation in these diseases. In particular, the capacity of Tie2 and inflammatory signalling pathways to cooperatively regulate inflammatory gene expression in ECs, FLS and macrophages suggests clinical benefit in simultaneously targeting both pathways, an idea already substantiated by pre-clinical studies in animal models of arthritis [14, 15].

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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