Targeting intracellular signaling pathways at the interface of T lymphocyte and innate immunity in immune-mediated inflammatory diseases
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Angiopoietin-2 promotes inflammatory activation of human macrophages and is essential for murine experimental arthritis


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Abstract

Background: Angiopoietin (Ang)-1 and Ang-2, and their shared receptor Tie2, are expressed in rheumatoid arthritis (RA) synovial tissue, but the cellular targets of Ang signaling and the relative contributions of Ang-1 and Ang-2 to arthritis are poorly understood.

Objectives: To determine the cellular targets of Ang signaling in RA synovial tissue, and the effects of Ang-2 neutralisation in murine collagen-induced arthritis (CIA).

Methods: RA and psoriatic arthritis (PsA) synovial biopsies were examined for expression of Tie2 and activated phospho (p)-Tie2 by quantitative immunohistochemistry and immunofluorescent double staining. Human monocyte and macrophage Tie2 expression was determined by flow cytometry and quantitative PCR. Regulation of macrophage intracellular signalling pathways and gene expression were examined by immunoblotting and ELISA. CIA was assessed in mice treated with saline, control antibody, prednisolone or neutralising anti-Ang-2 antibody.

Results: Expression of synovial Tie2 and p-Tie2 was similar in RA and PsA. Tie2 activation in RA patient synovial tissue was predominantly localised in synovial macrophages and was expressed by human macrophage. Ang-1 and Ang-2 stimulated activation of multiple intracellular signalling pathways, and cooperated with tumour necrosis factor to induce macrophage interleukin 6 and macrophage inflammatory protein 1α production. Ang-2 selectively suppressed macrophage thrombospondin-2 production. Ang-2 neutralisation significantly decreased disease severity, synovial inflammation, neo-vascularisation and joint destruction in established CIA.

Conclusions: We identify synovial macrophages as primary targets of Ang signaling in RA, and demonstrate that Ang-2 promotes the pro-inflammatory activation of human macrophages. Ang-2 makes requisite contributions to pathology in CIA, indicating that targeting Ang-2 may be of therapeutic benefit in the treatment of RA.
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Introduction

Angiogenesis of new vessels from existing vessels plays a pivotal role in mammalian development, and in the initiation and maintenance of chronic inflammatory diseases and tumour growth\(^1\). Receptor tyrosine kinase Tie2 ligation by angiopoietin (Ang)-1 and Ang-2 makes contributions to angiogenesis and blood vessel remodelling which are critically distinct from vascular endothelial growth factor receptor ligation\(^2\). Like vascular endothelial growth factor, Ang-1 and Tie2 are required for embryonic angiogenesis\(^3;4\). In mice, stable systemic Ang-1 levels maintain vascular stability and suppress inflammation-induced vascular permeability and oedema\(^5;6\). In mice, stable systemic Ang-1 levels maintain vascular stability and suppress inflammation-induced vascular permeability and edema\(^7\). By contrast, Ang-2 is up-regulated during inflammation, and promotes recruitment of neutrophils by activated endothelial cells (ECs)\(^8\).

Infiltration of solid murine and human tumours by Tie2-expressing monocytes/macrophages (TEMs) is critical in cancer establishment and growth\(^9-11\). Ang2 stimulation of TEMs potentiates tumour growth, in part by promoting immunosuppressive properties of TEMs, including interleukin 10 (IL-10) secretion\(^11-13\). Tie2 signalling is also thought to be important in chronic inflammatory diseases. In patients with rheumatoid arthritis (RA) and other forms of inflammatory arthritis, such as psoriatic arthritis (PsA), increased vascularity and new blood vessel formation promotes destruction of the affected joints by facilitating the influx of nutrients and inflammatory cells\(^14;15\). Histological changes in the synovial microvasculature precede local inflammation in patients presenting with mono-arthritis prior to fulfilment of classification criteria for RA, and in both RA and PsA, expression of angiogenic factors and EC activation markers are closely associated with, and suggested to be prognostic for disease activity and joint destruction\(^16-18\). Successful treatment of inflammatory arthritis is closely associated with decreases in systemic and local expression of angiogenic factors and endothelial activation markers\(^19-22\). This has indicated that modulation of angiogenesis may be useful in the treatment of RA, and direct targeting of newly formed blood vessels in animal models of RA prevents inflammation and joint destruction\(^23\). In addition, specific blockade of Tie2 signalling in vivo prevents angiogenesis and joint destruction in the murine CIA model of RA\(^24;25\). However, it is unclear whether Ang-1 and Ang-2 make independent contributions to pathology in inflammatory arthritis.

Ang-1, Ang-2 and Tie2 are readily detected in the synovial tissues of patients with RA and PsA\(^17;26-29\). Tie2 expression in synovial tissue is detected on ECs, stromal fibroblast-like synoviocytes (FLS) and macrophages, and in vitro, tumour necrosis factor (TNF) can induce EC Tie2 expression\(^24\). Ang-1 and Ang-2 are produced in situ by RA FLS, macrophages and ECs\(^17;27-29\). Ang-1 stimulation of Tie2 expressed on ECs and FLS can promote synovial angiogenesis and cartilage destruction in synovial tissue, respectively\(^24;30\). Effects of Ang-2 on RA synovial cells have not been examined, but
a role for Ang-2 in the pathology of human inflammatory arthritis is suggested by observations that synovial tissue and synovial fluid Ang-2 levels are highly elevated compared with Ang-1 in patients with RA and PsA\textsuperscript{17,28}. In addition, serum Ang-2 levels are associated with disease activity in recent onset of RA\textsuperscript{31}. Here, we examined the potential role of myeloid Tie2 signalling in RA, and the consequences of Ang-2 blockade in murine CIA.

\section*{Materials and Methods}

\subsection*{Patients, synovial biopsy acquisition and synovial tissue analysis}
Synovial biopsies were obtained by needle arthroscopy as previously described from clinically active joints of RA (n=20) and PsA (n=19) patients who fulfilled the American College of Rheumatology criteria for RA and the classification criteria for psoriatic arthritis, respectively\textsuperscript{32,34}. Patient characteristics for both cohorts have been previously described in detail\textsuperscript{35}. All patients gave their written informed consent prior to inclusion, and this study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam. Sections were processed for immunohistochemical and immunofluorescent analyses as previously described, detailed in the supplementary material\textsuperscript{36,37}.

\subsection*{Monocyte purification, macrophage differentiation, and analysis}
Human peripheral blood mononuclear cells were isolated from blood of healthy volunteer donors and RA patients by Ficoll, and monocytes were either used immediately or differentiated into macrophages as previously described\textsuperscript{38}. Full details of monocyte isolation, macrophage culture, differentiation, characterisation by flow cytometry and stimulation and analysis by immunoblotting and ELISA are described in the supplementary material.

\subsection*{Animals, and induction and assessment of CIA}
DBA/1 male mice were purchased from either Harlan (Horst, The Netherlands) or Jackson Laboratory (Bar Harbor, Maine, USA). Animals were housed in specific pathogen-free conditions at Alderley Park, Astra Zeneca, in compliance with Home Office Regulations (UK). All animal experiments were approved by the Animal Welfare and Ethics Committee (Alderley Park). Mice (males, 6–7 weeks of age) were immunised by tail base injection on day 0 with an emulsion of rat type II collagen (100 µg, MD Biosciences Gmbh, Zurich, Switzerland) and complete freund’s adjuvant (1 mg/ml, Difco), followed by boosting with Staphylococcus enterotoxin B (SEB; 30 µg, Toxin Technology) in incomplete freund’s adjuvant (Sigma Aldrich, St. Louis, MO) on day 21. The mice were examined daily from day 22 until sacrifice for clinical signs of arthritis.
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Arthritis severity was assessed in a blinded manner, using a semiquantitative scoring system: 0– normal; 1– one digit affected, or erythema of paw; 2– slight swelling and/or erythema; 3– pronounced swelling; 4– immobilisation of paw/ankylosis. Each limb was scored independently, allowing for a cumulative maximal score of 16 for each mouse. A clinical score of two on a single paw (minimally) triggered treatment. Vehicle (PBS, Sigma Aldrich), human IgG1 isotype control antibody at 10 mg/kg (Sigma Aldrich) and anti-Ang-2 antibody (Clone 3.19.3) at 0.1, 1 and 10 mg/kg were administered intraperitoneally every three days, and prednisolone was administered orally once daily at 3 mg/kg (Sigma Aldrich) (n=10 mice per treatment group).

Statistical analyses
For in vivo studies, analysis was performed using analysis of variance (ANOVA) with post hoc Dunnetts analysis on Windows Graphpad Prism 4. For in vitro experiments, analysis was performed using Windows Graphpad Prism 5 (GraphPad Software). Flow cytometry and ELISA results were expressed as the mean ± SEM. Potential differences between samples or scores were analysed by Student’s t-test or non-parametric Mann–Whitney U or Kruskal–Wallis tests, as appropriate. p Values ≤0.05 were considered significant.

Results
Tie2 is activated in RA synovial tissue macrophages
We first examined the expression and activation status of Tie2 in RA and PsA synovial tissue. Specific reactivity of anti-Tie2 and p-Tie2 antibodies was readily detected in both RA and PsA synovial tissue, while negative control antibodies failed to stain synovial tissue (figure 1A). Staining with Tie2 and p-Tie2 antibodies was prominently observed in the intimal lining layer, perivascular regions and synovial sublining cellular infiltrates (figure 1A). Similar levels of both Tie2 and p-Tie2 expression were observed in RA and PsA synovial tissue, as measured by digital imaging analysis (figure 1B). Immunofluorescent double-staining experiments demonstrated that Tie2 was not expressed in CD3+ T lymphocytes in RA synovial tissue, but was occasionally detectable on VWF+ ECs and CD55+ FLS (figure 1C, left panels). However, the most prominent expression of Tie2 was observed in CD68+ and CD163+ synovial macrophages. Remarkably, p-Tie2 staining was almost exclusively restricted to CD68+ and CD163+ macrophages infiltrating RA synovial tissue, and was seldom observed in ECs or FLS (figure 1C, right panels).

Tie2 is functionally expressed on human macrophages
Consistent with published studies, Tie2 was readily detected on the surface of freshly isolated human CD14+ peripheral blood mononuclear cells, and on peripheral blood-derived human macrophages (figure 2A, B). Macrophage Tie2 surface expression
Figure 1. Tie2 is expressed and activated in rheumatoid arthritis (RA) synovial tissue macrophages. (A) Immunohistochemical analyses of RA synovial tissue stained with control rabbit antibodies, anti-Tie2 antibodies, normal rabbit serum and anti-p-Tie2 hyperimmune serum. Original magnification, ×100 (upper and middle panels) and ×400 (lower panels). (B) Quantitative analysis of Tie2 and p-Tie2 staining in synovial tissue. Synovial sections from 20 RA and 19 psoriatic arthritis (PsA) patients were stained with antibodies against Tie2 and p-Tie2 antibodies as above, and the integrated optical density (IOD)/mm² corrected for cellularity calculated by digital image analysis. Data is 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. (C) Immunofluorescent staining of RA synovial tissue with anti-Tie2 (green, left panels) and anti-phospho (p)-Tie2 Ab (green, right panels) in combination with anti-CD3, -CD55, -CD68, -CD163 and -VWF Abs (all red). Colocalisation is visualised in yellow. Original magnification, ×100. All stainings are representative of stainings performed on synovial tissue from 5 RA patients.
Figure 2. Tie2 is expressed by human peripheral blood-derived monocytes and macrophages. (A) Viable monocytes (upper panels) and macrophages (lower panels) were gated based on side scatter (SSC) and forward scatter (FSC) (left panels) by flow cytometry and stained with CD14 and either isotype-matched control IgG (middle panels) or Tie2 Abs (right panels). Dot blots shown are representative of experiments performed on 6 individual blood donors. (B) Staining of monocytes and macrophages with control IgG (gray) and Tie2 (empty) Abs shown in A presented as histograms. (C) Quantification of relative Tie2 surface expression (geomean, upper panel) and mRNA expression (lower panel) in monocytes and macrophages. Surface expression values are mean ± SEM. *p<0.05 versus monocytes. For quantitative analysis of Tie2 mRNA expression in human macrophages, monocytes, RA fibroblast-like synoviocytes (FLS) and human umbilical vein endothelial cells (HUVEC), data are assessed as the ratio of mRNA expression of Tie2 to mRNA expression of glyceraldehydes-3-phosphate dehydrogenase and expressed as the fold difference relative to macrophages. Values for macrophage mRNA expression are normalised to 1. Bars represent the mean ± SEM of 3–5 independent samples of each cell type. (D) Comparative surface expression of Tie2 in healthy donor (HD) and RA patient peripheral blood CD14+ monocytes and differentiated macrophages. Data are presented as in C.
Figure 3. Macrophage Tie2 stimulation activates intracellular signalling pathways and enhances tumour necrosis factor (TNF) induced interleukin 6 (IL-6) production. (A) Immunoblot analyses of cellular lysates obtained from macrophages stimulated for the indicated times (min) with Ang-1 (left panels) or Ang-2 (right panels) for expression and phosphorylation (p) of the indicated signalling proteins. All data shown are from independent immunoblots performed on macrophage lysates obtained from a single donor and are representative of three individual experiments.
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was reduced by approximately 25% in comparison with monocytes (p<0.05) (figure 2C, upper panel), again in agreement with published studies. Tie2 mRNA expression was highest in human umbilical vein ECs and RA FLS, followed by monocytes and macrophages (figure 2C, lower panel). No differences were observed in Tie2 surface expression when comparing monocytes and macrophages obtained from healthy donor or RA patient peripheral blood (figure 2D).

Despite the relatively low levels of Tie2 expression on macrophages, Ang-1 and Ang-2 stimulation could generate cellular response, as evidenced by activation of macrophage intracellular signalling pathways and gene expression. Ang-1 stimulation transiently increased phosphorylation of ERK-1/2, p38 and JNK mitogen-activated protein kinases, and PKB (figure 3A). Ang-2 activated the same signalling pathways, albeit with delayed kinetics and decreased magnitudes of activation. In particular, JNK activation was barely detectable following Ang-2 stimulation. Ang-1, but not Ang-2, also induced transient phosphorylation of IκBα.

Stimulation with either Ang-1 or Ang-2 alone failed to induce macrophage IL-6 production compared with unstimulated macrophages (figure 3B). However, in the presence of TNF (10 ng/ml), Ang-1 (200 ng/ml) significantly increased IL-6 production compared with TNF treatment alone (p<0.05). Similar enhancing effects were observed with Ang-2 (p<0.01), although lower concentrations of Ang-2 (20 ng/ml) also significantly enhanced IL-6 production. The effects of Ang-1 and Ang-2 on TNF-induced IL-6 production were selective, as both failed to influence LPS-induced IL-6 production. When lower concentrations of TNF were used, Ang-1 synergistically enhanced IL-6 production (p<0.01), while only a trend towards enhanced IL-6 production was observed with Ang-2 (figure 3C). Ang-1 co-stimulation with 1 ng/ml TNF created a biological window in which to identify specific Tie2 signalling pathways contributing to IL-6 production. Pre-incubation of macrophages with pharmacological inhibitors of NF-κB (BAY 11-7082) and MEK/ERK (U0126) but not PKB, JNK or p38 (SB23580) significantly reduced the capacity of Ang-1 to enhance IL-6 production (figure 3D). Under these experimental conditions, Ang-1 and TNF cooperatively enhanced macrophage NF-κB, ERK and PKB activation (see supplementary figure 1).
Ang-1 and Ang-2 differentially regulate macrophage angiogenic factor expression

Supernatants from experiments shown in figure 3 were pooled and screened using a proteomic array detecting defined secreted angiogenic factors (data not shown). In independent validation ELISA experiments, MIP-1α and IL-8 were not directly induced by stimulation with Ang-1 or Ang-2 alone (figure 4A). However, both Ang-1 and

**Figure 4.** Macrophage Tie2 stimulation regulates production of angiogenic factors. Analyses of (A) MIP-1α and IL-8, (B) matrix metalloproteinase (MMP)-9 and TIMP-1, and (C), and TSP-1 and TSP-2 production in tissue culture supernatants of granulocyte macrophage colony-stimulating factor (GM-CSF)-differentiated macrophages after 24 h incubation in medium alone, TNF (10 ng/ml) or lipopolysaccharide (LPS) (1 µg/ml) in the absence (white bars) or presence of Ang-1 (200 ng/ml, gray bars) or Ang-2 (200 ng/ml, black bars). Bars represent the means and SEM of 6 independent experiments. *p<0.05, **p<0.01 versus cells not exposed to Ang-1 or Ang-2.
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Ang-2 cooperated with TNF to stimulate MIP-1α production ($p<0.01$). Ang-1 ($p<0.01$) and Ang-2 ($p<0.05$) also synergised with lipopolysaccharide (LPS) to induce MIP-1α production. By contrast, IL-8 production in response to TNF or LPS was unaffected by Tie2 signalling. Constitutive matrix metalloproteinase (MMP)-1 (data not shown), MMP-9 and TIMP-1 production (figure 4B) were unchanged following TNF or LPS stimulation, alone or in combination with Ang-1 or Ang-2, as was secretion of TSP-1 (figure 4C). However, Ang-2 selectively suppressed spontaneous TSP-2 production, and TNF- and LPS-induced TSP-2 production ($p<0.05$). Together, these results indicate that macrophage Tie2 signalling, particularly following engagement by Ang-2, can promote a pro-inflammatory environment by enhancing cytokine and chemokine production while suppressing constitutive production of TSP-2, a natural inhibitor of MMPs and tissue retention of inflammatory cells.

Neutralization of Ang-2 is protective in murine CIA

To determine the therapeutic potential of blocking Ang-2 function in inflammatory arthritis, we evaluated a neutralising, high affinity, fully humanised anti-Ang-2 antibody, 3.19.3, which prevents human and murine Ang-2 signalling to Tie2 in the murine CIA model of RA. Treatment of mice was initiated 24 h after the clinical onset of arthritis by administration of saline, increasing dosages of 3.19.3, isotype-matched control antibody (10 mg/kg), or prednisolone. Mice treated with isotype-matched control antibody and saline showed no significant differences in disease severity throughout the course of the experiment (figure 5A). However, clear clinical improvements were observed in mice treated with 1.0 and 10 mg/kg 3.19.3 and prednisolone. Analyses of the area under the curve (AUC) for clinical scores for the course of the experiment demonstrated significant suppression of disease severity in mice treated with 1 (53±7% suppression, $p<0.05$), 10 mg/kg (44±9% suppression, $p<0.05$) and prednisolone (78±7% suppression, $p<0.05$). Histological analyses of the hind paws of mice were performed to gain insight into the cellular mechanism(s) by which Ang-2 blockade protected against arthritis. H&E staining of joints revealed that 3.19.3 reduced multiple histopathological parameters of disease in the CIA model, including synovial infiltration and cartilage erosion (figure 5B). Semiquantitative analyses demonstrated that 3.19.3 administration conferred improvements in synovitis, synovial hyperplasia, synovial fibrosis, pannus formation (periosteal infiltration) and periostitis (supplementary figure 2). Using a global combined histological score, an inhibition of arthritic pathology was observed which mirrored improvements in clinical scores (figure 5C).

Tissues were stained with anti-CD31 Abs to visualise the presence of newly formed blood vessels (figure 6A). At dosages of 1 and 10 mg/kg, 3.19.3 reduced microvessel density by approximately 50% ($p<0.005$) (figure 6B). Joint sections were also assessed for lymphatic microvessel density by immunohistochemical staining with anti-LYVE-1 Ab followed by counting of microvessel LYVE-1 aggregates. Compared with isotype control
antibody, 3.19.3 significantly reduced lymphatic microvessel density by >80% at all dosages tested (p<0.005) (figure 6C). A significant reduction in macrophage cellularity was observed at 1 and 10 mg/kg 3.19.3, although no inhibition was observed at either 0.1 mg/kg 3.19.3 or 10 mg/kg isotype control antibody (figure 6D).
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**Discussion**

In this study, we demonstrate that Tie2 is functionally expressed on activated pro-inflammatory macrophages, and identify synovial macrophages as primary targets of Tie2 signalling in RA. Ang-2, alone, or in combination with TNF, can contribute to the inflammatory activation of macrophages, and as demonstrated by in vivo neutralisation experiments, Ang-2 is required for pathology in murine CIA. Our observation that Tie2 activation in RA synovial tissue is largely restricted to infiltrating macrophages is striking,
as it necessitates reassessment of the interplay between angiogenic factors, innate immunity and pathology in chronic inflammatory disease. Consistent with previous studies, we observed that Tie2 was expressed by ECs, FLS and macrophages in RA synovial tissue. However, ECs and FLS expressing p-Tie2 were only rarely detected. By contrast, p-Tie2 was observed in the majority of RA synovial macrophages. Preferential activation of macrophages may arise from differential proximity of this cell population to other synovial cells producing Ang-1 and Ang-2, which requires further experimental consideration, other cell populations in synovial tissue may express relatively higher levels of inhibitory Tie1, or macrophages might differ from other synovial cell populations in their sensitivity or exposure to soluble inhibitory Tie1 splice variants.

Direct and critical integration of angiogenic signalling with innate immune response components has recently been identified in tumour biology. Here, recruitment of TEMs, suggested to represent a distinct myeloid lineage commitment, is necessary for the establishment and growth of murine and human solid tumours. Prominent secreted gene products expressed by TEMs include cathepsin B, MMP-9 and IL-10, and expression of these factors, important for angiogenesis and immune regulation, is reinforced by Ang-2 stimulation. Our studies demonstrate that Tie2 expression on differentiated human macrophages can also contribute to pro-inflammatory activation of these cells via induction of IL-6 production, which pleiotropically supports cellular activation and survival in RA. Macrophage Tie2 stimulation also enhances production of MIP-1α, a chemokine expressed at high levels in the RA synovium and shown to play an essential inflammatory role in animal models of RA. Ang-1 or Ang-2 stimulation alone was unable to generate macrophage IL-6 or MIP-1α expression, but instead synergised with TNF (and in the case of MIP-1α, also LPS) to support gene expression, a co-stimulatory property strikingly similar to the combined effects of Tie2 ligation and TNF stimulation during EC activation. This is reinforced by our observation that Ang-1, and to a lesser extent Ang-2, can increase IL-6 production in the presence of low concentrations of TNF. Lastly, we observe a specific role for Ang-2 in dampening both constitutive and agonist-induced macrophage TSP-2 production. TSP-2 broadly orchestrates angiogenic remodelling, via the promotion of EC apoptosis, interference with integrin ligand binding, and inhibition of MMPs. In mice lacking TSP-2, defects are observed in extracellular matrix remodelling during angiogenesis, associated with increased MMP-9 deposition and gelatinase activity. Overexpression of TSP-2 inhibits vascularisation, inflammation and lymphocyte accumulation in the human RA-severe combined immunodeficiency model of RA. The ability of Ang-2 to stimulate macrophage chemokine production, while simultaneously suppressing TSP-2 output, may promote inflammatory leucocyte recruitment and retention, and facilitate MMP-driven tissue destruction in RA.

Gene therapy strategies delivering local expression of soluble Tie2 inhibit both angiogenesis and pathology in CIA. Using 3.19.3, a neutralising Ang-2 antibody, we demonstrate a specific and critically important pro-inflammatory role for Ang-2 in
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disease progression of established clinical arthritis in murine CIA. Ang-2 neutralisation blocked not only new vessel formation, but also reduced the number of inflammatory cells infiltrating arthritic joints, both of which are processes pivotal to the development of arthritis\textsuperscript{1,46}. The clinical, histological and radiological protection provided by Ang-2 blockade closely recapitulates effects observed following adenoviral delivery of soluble Tie2 receptor during murine CIA\textsuperscript{25}. That synovial macrophages are primary targets of Ang signalling in RA is noteworthy from the perspective of therapeutic application. Synovial macrophage numbers and cytokine production are tightly associated with disease activity, and decreases in synovial sublining CD68\textsuperscript{+} macrophages precede and predict effective clinical responses to therapy\textsuperscript{47,48}. Therefore, therapies specifically targeting macrophage retention and activation in the synovium are thought to be of future clinical benefit\textsuperscript{46}. The capacity of Ang-2 to support both these features, and recent findings that serum levels of Ang-2 are associated with inflammation and disease activity in recent onset RA, warrants further assessment of the unique contributions of macrophage Tie2 signalling to RA, and comparative analysis of Ang-1 blockade in inflammatory arthritis\textsuperscript{17,27,29,31}.

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References


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Supplemental Methods

Immunohistochemistry
Biopsy sections from each patient were cut with a cryostat (5 µm) and mounted on glass slides (Starfrost). The tissues were then fixed with acetone, and endogenous peroxidase activity blocked by immersion in 0.3% hydrogen peroxide and 0.1% sodium azide in PBS. The slides were incubated overnight at 4°C with optimized dilutions of primary antibodies diluted in 1% BSA/PBS. Primary antibodies used in this study were polyclonal rabbit antibodies specific for Tie2 (Santa Cruz Biotechnology) and FITC (Thermo Fisher Scientific), anti-phosphorylated (p)-Tie2 rabbit hyperimmune serum (Calbiochem/Merck Chemicals Ltd.), and normal rabbit serum. Equivalent concentrations of anti-FITC rabbit polyclonal antibodies, or anti-FITC antibodies diluted in normal rabbit serum, were used as negative controls. Sections were washed with PBS and incubated with swine anti-rabbit-horseradish peroxidase (HRP)-conjugated Ab (Dako), followed by sequential incubation with biotinylated tyramide and streptavidin-HRP, and development with aminoethylcarbazole (Sigma-Aldrich). Slides were counterstained with Mayer’s hematoxylin and mounted in Kaiser’s glycerol gelatin (Merck).

Immunofluorescence
To detect specific cell populations expressing Tie2 and p-Tie2, synovial tissue biopsies were incubated overnight at 4°C with primary antibodies against Tie2 or p-Tie2, followed by Alexa 488-conjugated goat anti-rabbit antibodies (Molecular Probes Europe). After washing, sections were sequentially incubated with specific antibodies recognizing CD3 (to detect T lymphocytes), von Willebrand factor (VWF) (ECs), CD68 and CD163 (macrophages), followed by Alexa 596-conjugated goat anti-mouse antibodies (all from Molecular Probes Europe). Samples were then mounted in Vectashield (H-1000; Vector), examined with a fluorescence microscope (Leica DMRA) and CCD camera, and data acquired using Image-Pro Plus software (Media Cybernetics, Dutch Vision Components).

Monocyte purification, macrophage differentiation, and flow cytometry
Monocytes were either used immediately or differentiated into macrophages by culture at 5 x 10⁵ cells per well in 24-well tissue culture plates in IMDM (Invitrogen) supplemented with 1% FCS. Non-adherent cells were removed after one hour by washing with IMDM/1% FCS, and adherent cells cultured in IMDM/10% FCS supplemented with 100 µg/ml gentamycin (Invitrogen), in the absence or presence of granulocyte macrophage colony-stimulating factor (GM-CSF) (5 ng/ml, Biosource International) for 7 days. On day 4, the medium was refreshed by replacing half of the medium with fresh IMDM/10% FCS supplemented with GMC-CSF. Monocyte and macrophage purity was assessed by staining with anti-CD14 PerCP-Cy5.5 (BD Biosciences) and anti-CD68-FITC (Dako)
monoclonal antibodies and flow cytometry (FACSCalibur, BD Biosciences). Expression of Tie2 with APC-conjugated anti-Tie2 (R&D Systems) was determined on CD14<sup>+</sup> cells, and an isotype-matched APC-conjugated IgG1 Ab (BD Biosciences) control was used in all Tie2 staining procedures. Confirmation of Tie2 expression by real-time PCR expression was performed as detailed below.

**Real-time PCR**

Cells were harvested, washed with cold PBS, and total RNA extracted using a GenElute RNA isolation kit (Sigma-Aldrich). Total RNA was reverse-transcribed using SuperScript<sup>™</sup> II RT (Invitrogen). Duplicate PCR reactions were performed using SYBR green (Applied Biosystems) with an ABI Prism® 7000 sequence detection system (Applied Biosystems). cDNA was amplified using specific primers for Tie2 (forward, sequence ACAATGGTGTCTGCCATGAA; reverse, TTCACAAGCCTTCTCACACG) (Invitrogen). All PCR data were normalized to the expression of GAPDH, used as an internal control. PCR data were obtained as Ct values and the mean of the duplicate Ct values of each sample was calculated. The difference between the Ct value of Tie2 and GAPDH (ΔCt) was used to determine relative Tie2 expression levels.

**Immunoblotting**

Macrophages were left unstimulated or stimulated for up to 60 minutes in the absence or presence of recombinant human Ang-1 or Ang-2 (200 ng/ml, R&D Systems). Equivalent numbers of macrophages were lysed in 1x Laemmlí’s buffer and total cell lysates were heated for 10 minutes at 90°C and then loaded on 4-12% gradient Bis-Tris SDS NuPAGE® gels (Invitrogen). After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad Laboratories) using a semi-dry transfer apparatus (Invitrogen). Membranes were washed in Tris-buffered saline (TBS) (pH 8.0) containing 0.05% Tween-20 (Bio-Rad) (TBS/T), blocked in TBS/T containing 2% milk (Bio-Rad) and incubated overnight at 4°C in primary antibodies diluted in TBS/T. All primary antibodies were purchased from Cell Signaling Technology. Primary antibodies used were antibodies specific for p-IκBα, IκBα, p-protein kinase B (pPKB) (Ser473), PKB, p-extracellular regulated kinase (pERK) 1/2, ERK 1/2, p-p38, p38, p-c-jun N-terminal kinase (pJNK), and JNK. Following washing, membranes were incubated in TBS/T containing IRDye infrared secondary Ab (LI-COR/Westburg), developed with Odyssey system (LI-COR) and visualized using Odyssey application software version 3 (LI-COR).

**Measurement of cytokine production**

Macrophages were left unstimulated or stimulated for 24 hours with TNF or lipopolysaccharide (LPS) (both from Sigma-Aldrich), in the absence or presence of increasing concentrations of Ang-1 or Ang-2 (2, 20, 200 ng/ml). Where indicated, cells were pre-incubated for 1 hour with BAY 11-7082 (10 μM), Akt inhibitor VIII (10 μM), JNK
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Inhibitor II (20 µM), SB23580 (10 µM) or U0126 (10 µM) (all from Calbiochem) prior to stimulation. Cell-free supernatants were collected and stored at -20°C until analyzed by ELISA for IL-6, IL-8, (both PelKine Compact™ ELISA kits, Sanquin Reagents) macrophage inflammatory protein-1α (MIP-1α), tissue inhibitor of metalloproteinases-1 (TIMP-1), MMP-9, TSP-1, and TSP-2 (all from R&D Systems) as per manufacturer’s protocols.

Histopathology

Following animal sacrifice, hind paws were fixed, followed by decalcification and embedding in paraffin. Sagittal serial sections were cut and stained with haematoxylin and eosin (H&E). Sections were evaluated for the presence of cellular infiltration (synovitis), synovial hyperplasia, fibrosis, pannus formation, periostitis, and bone erosions a using semi-quantitative scoring system: 0- normal, 1- minimal, 2- moderate, and 3- severe. Individual scores for each parameter, with the exception of bone erosions, were summed for individual mice to obtain overall histological scores. Serial sections were also stained for the vascular marker CD31 and LYVE-1. For CD31 staining, sections were washed in 0.05% PBS/T, and stained with rat anti-mouse CD31 (Clone MEC13.3, BD Pharmingen) or rat IgG2a isotype control for 1 hour at room temperature. Sections were then washed and stained with biotinylated rabbit anti-rat IgG (1:200 dilution, DAKO) for 30 minutes followed by staining with TSA Biotin system (Perkin Elmer) and DAB (DAKO). Five serial sections of the tibio-talus synovium were taken and CD31+ vessels within the superficial and intermediate synovium were counted. The difference in tissue volumes between joints from diseased and control mice were normalized to 500µm². Individual CD31+ cells and bone associated CD31+ cells were not counted. For LYVE-1, 5µm tissue sections were cut, removed from paraffin, and subjected to heat-mediated antigen retrieval using Target Antigen Retrieval fluid (Dako). Five serial sections of the tibio-talus synovium were stained with anti-LYVE-1 Ab (Ab 14917, Abcam) on a DakoCytomation Envision+ System (Dako), and vessels within the superficial and intermediate synovium expressing LYVE-1 were quantified as for CD31 above. Macrophage cellularity of synovial tissue in each hind paw was determined by semi-quantitative scoring by a cellular pathologist for the presence of hypertrophic ‘blastic-type’ macrophages within the synovium: 0 - no pathology, 1- mild macrophage cellularity, 2- moderate macrophage cellularity, and 3 - severe macrophage cellularity.
Supplemental Figure 1 Ang-1 cooperates with TNF to activate intracellular signaling pathways in macrophages. Immunoblot analyses of cellular lysates obtained from macrophages stimulated for the indicated times (min) with medium alone, or TNF alone or in combination with Ang-1 (200 ng/ml) for expression and phosphorylation (p) of the indicated signaling proteins. All data shown are from independent immunoblots performed on macrophage lysates obtained from a single donor and are representative of 3 individual experiments.
Supplemental Figure 2  Individual histopathological parameters of disease activity during CIA in each treatment group. Semi-quantitative scores for each parameter were established based on criteria detailed in Materials and Methods. Data are mean ± SEM for each group (n = 10 animals per group).