Angiogenesis and Blood Vessel Stability in Inflammatory Arthritis

Aisling Kennedy,1 Chin Teck Ng,1 Monika Biniecka,1 Tajvur Saber,1 Cormac Taylor,2 Jacintha O’Sullivan,1 Douglas J. Veale,1 and Ursula Fearon1

Objective. To assess blood vessel stability in inflammatory synovial tissue (ST) and to examine neural cell adhesion molecule (NCAM), oxidative DNA damage, and hypoxia in vivo.

Methods. Macroscopic vascularity and ST oxygen levels were determined in vivo in patients with inflammatory arthritis who were undergoing arthroscopy. Vessel maturity/stability was quantified in matched ST samples by dual immunofluorescence staining for factor VIII (FVIII)/α-smooth muscle actin (α-SMA). NCAM and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) were examined by immunohistochemistry. Angiogenesis was assessed in vitro, using human dermal endothelial cells (HDECs) in a Matrigel tube formation assay.

Results. A significant number of immature vessels (showing no pericyte recruitment) was observed in tissue from patients with inflammatory arthritis (P < 0.001), in contrast to osteoarthritic and normal tissue, which showed complete recruitment of pericytes. Low in vivo PO2 levels in the inflamed joint (median [range] 22.8 [3.2–54.1] mm Hg) were inversely related to increased macroscopic vascularity (P < 0.04) and increased microscopic expression of FVIII and α-SMA (P < 0.04 and P < 0.03, respectively). A significant proportion of vessels showed focal expression of NCAM and strong nuclear 8-oxodG expression, implicating a loss of EC–pericyte contact and increased DNA damage, levels of which were inversely associated with low in vivo PO2 (P = 0.04 for each comparison). Circulating cells were completely negative for 8-oxodG. Exposure of HDEC to 3% O2 (reflecting mean ST in vivo measurements) significantly increased EC tube formation (P < 0.05).

Conclusion. Our findings indicate the presence of unstable vessels in inflamed joints associated with hypoxia, incomplete EC–pericyte interactions, and increased DNA damage. These changes may further contribute to persistent hypoxia in the inflamed joint to further drive this unstable microenvironment.

Inflammatory arthritides are chronic progressive diseases that are characterized by proliferation of the synovial tissue (ST), leading to the degradation of articular cartilage and subchondral bone. Angiogenesis is an early event in the inflammatory joint that is important in enabling activated monocytes to enter the synovium and expand it into a pannus via endothelial cells (ECs) by active recruitment (1,2), resulting in cartilage degradation and bone destruction. Previous studies have demonstrated increased angiogenesis and distinct vascular morphology in the synovial membrane (SM) of inflammatory arthritis during early disease (3), which is associated with increased differential expression of proangiogenic factors in synovial fluid (SF) and ST (4,5).

ECs receive cues from proinflammatory molecules that initiate mitosis, migration, and organization of primitive angiotubes (6–8), leading to the formation of a mature vasculature. Vascular endothelial growth factor (VEGF) is a main angiogenic “on” switch, which acts early in vascular morphogenesis, stimulating EC prolif-
eration and migration. However, in the presence or absence of angiopoietin 1/2 (Ang-1/2), placenta growth factor, platelet-derived growth factor (PDGF), or transforming growth factor β1 (TGFβ1), blood vessels may undergo further remodeling, resulting in either immature or mature, stable vessels (6,9,10). The acquisition of a pericyte layer ends the “plastic” state, stabilizing the vessel (6,11,12). Vessel stability is also dependent on EC–pericyte interactions and perivascular extracellular matrix (ECM) components, such as the immunoglobulin neural cell adhesion molecule (NCAM). NCAM deficiency results in an unstable EC–pericyte interaction and vessel survival (11,13,14). This process is dependent on cell survival signals, which may influence nuclear instability events, such as telomere length shortening induced by high levels of oxidative stress (15).

Hypoxia is defined as cellular demand for molecular oxygen exceeding vascular supply, leading to bioenergetic crisis. Increases in uniform blood flow in the pannus fail to restore tissue oxygen levels, causing chronic, persistent cell infiltration and invasion. Previous studies have shown that the oxygen level in rheumatoid arthritis (RA) ST is reduced compared with the level in normal controls, and low oxygen levels have been described in tenosynovium in patients with tendon rupture (16–18). Hypoxia-inducible factor 1α (HIF-1α), which is a key transcription factor in the cellular response to hypoxic conditions, is also up-regulated in RA synovium (19), and in vitro, it is involved in angiogenesis, migration, cell survival pathways, reactive oxygen species (ROS), glycolysis, and matrix degradation (16,18,20,21).

Oxidative stress occurs when a disturbance develops in the balance between the production of ROS and antioxidant defenses. Alterations in mitochondrial oxygen sensing can result in increased release of ROS, coupled with a compromised antioxidant defense system (22). Excessive production of ROS can damage biologic molecules, including DNA, lipids, and proteins. The DNA adduct 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is formed by the reaction of the OH radical with the DNA guanine base. It is a promutagenic lesion that mispairs with adenine, leading to GC to TA transversion. This oxidative DNA damage is repaired primarily by base excision repair pathways incorporating the glycosylates OGG1, MYH, and NUDT1 (16,23). Disturbance of this system can lead to oxidative damage, which is associated with many pathologic conditions, such as atherosclerosis, diabetes mellitus, and cancer (24,25). However, the pathophysiologic role of oxidative damage in inflammatory arthritis remains unclear. Previous studies have shown that SF T cells have decreased levels of the antioxidant glutathione (26) and that oxidative conditions in RA ST are associated with a higher incidence of p53 mutations (27).

In this study, we quantified blood vessel maturity and stability, in vivo Po2 levels, and the level of oxidative damage in the inflamed ST. Furthermore, we examined the relationship of vascularity, blood vessel stability, and DNA damage to the level of oxygen in the inflamed tissue measured using a novel Po2 probe.

**MATERIALS AND METHODS**

**Arthroscopy and ST collection.** Patients with active inflammatory arthritis (RA or psoriatic arthritis [PsA]) or osteoarthritis (OA) were recruited from rheumatology clinics prior to starting therapy with biologic agents. Healthy control subjects were also recruited. The majority of the patients had never taken disease-modifying antirheumatic drugs or steroids. Twelve patients were taking methotrexate, 1 patient was taking sulfasalazine, and 1 patient was taking azathioprine. Patients were diagnosed as having RA according to the American College of Rheumatology (formerly, the American Rheumatism Association) criteria (28) and as having PsA according to previously defined criteria (29). The study was approved by the St. Vincent’s University Hospital Medical Research and Ethics Committee, and all patients provided fully informed written consent. Clinical assessments included tender and swollen joint counts, rheumatoid factor status, anti–cyclic citrullinated peptide (anti-CCP) antibody status, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Disease Activity Score in 28 joints (DAS28), and global health, by visual analog scale (VAS). Arthroscopy of the inflamed knee was performed under local anesthetic using a Wolf 2.7-mm needle arthroscope, as previously described (3). SM vascularity was assessed macroscopically by direct visualization and quantified as validated previously (3), using a VAS (0–100 mm).

**Measurements of Po2 levels.** A Licox combined Po2 and temperature probe (CCI.P1; Integra Life Sciences, Plainsboro, NJ) was used to obtain Po2 levels. Following the first biopsy, the novel Licox Po2 probe (Integra Life Sciences) was introduced via a 22-gauge needle and positioned into the biopsy pocket under direct visualization, allowing direct tissue Po2 (tPo2; mm Hg) measurements in the SM sublining layer. Biopsy specimens were then obtained and embedded in OCT compound (Tissue-Tek; Sakura, Zoeterwoude, The Netherlands) or embedded in paraffin for immunohistochemical analysis.

**Blood vessel maturity.** Seven-micrometer OCT sections were placed on glass slides coated with 2% 3-aminopropyltriethoxysilane (Sigma-Aldrich Ireland, Dublin, Ireland) and dried overnight at room temperature. Sections were stored at −80°C until required for staining. Tissue sections were allowed to reach room temperature, fixed in acetone for 10 minutes, and air dried. Nonspecific binding was blocked using 10% casein in phosphate buffered saline (PBS) for 10 minutes at room temperature. Slides were washed in PBS for 5 minutes. To examine the maturity of blood vessels, the presence of immature and mature blood vessels was assessed. Dual immunofluorescence staining was initially performed for factor VIII (FVIII/α-smooth muscle actin (α-SMA; a pericyte marker, indicating vessel maturity). Slides were stored at −80°C until required for staining. Tissue sections were allowed to reach room temperature, fixed in acetone for 10 minutes, and air dried. Nonspecific binding was blocked using 10% casein in phosphate buffered saline (PBS) for 10 minutes at room temperature. Slides were washed in PBS for 5 minutes.
were coinubcated with a primary antibody against rabbit anti-FVIII and mouse anti-α-SMA (Dako, Glostrup, Denmark) and an appropriate IgG isotype-matched control for 1 hour in a humidified chamber. Immunofluorescent sections were washed in PBS for 5 minutes and incubated with a Cy2/Cy3-conjugated secondary antibody (Jackson Immuno-Research, Suffolk, UK) for 30 minutes. Slides were washed in PBS for 5 minutes and counterstained with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (1:1,000; Sigma, St. Louis, MO) for 10 minutes. Sections were mounted with anti-fade (Molecular Probes, Eugene, OR) and assessed by immunofluorescence microscopy. Fluorescent images were captured using a confocal microscope using a multi-track filter program for Cy2, Cy3, and DAPI excitations and emissions. Images were processed using LSM imaging software (Zeiss, Oberkochen, Germany). In a subgroup of patients, serial sections were stained with a primary antibody against mouse anti-FVIII and rabbit anti-lymphatic vessel endothelial marker 1 (anti-LYVE-1; Abcam, Cambridge, UK) to differentiate lymphatic from pan vessels.

We also examined blood vessel maturation using another double staining kit (EnVision G2 double-stain system, catalog no. K5361; Dako). This protocol was carried out according to the recommendations of the manufacturer, and positive staining was detected using diaminobenzidine/permanent red secondary antibodies. Blood vessel maturation was quantified by the number of blood vessels per high-power field (hpf) coexpressing FVIII/α-SMA compared with those expressing only FVIII. Five random fields were analyzed.

NCAM and 8-oxodG expression. For NCAM expression, cryostat sections were allowed to reach room temperature, fixed in acetone for 10 minutes, and air dried as described above. Staining with 8-oxodG was performed on paraffin-embedded sections. Sections were baked at 90°C for 30 minutes, deparaffinized in xylene, and rehydrated in alcohols and deionized water. Antigen retrieval was performed using 1,500 ml of citrate solution. This solution was heated in a pressure cooker with the lid off for 20 minutes in an 800V microwave. Slides were placed in a pressure cooker with the lid sealed and were heated at full power until the pressure valve popped (5–7 minutes). Slides were removed from the pressure cooker once the pressure had equalized and were washed in PBS and 0.05% Tween 20 for 5 minutes. For both cryostat and paraffin sections, nonspecific binding was blocked using 10% casein in PBS for 10 minutes at room temperature. A routine 3-stage immunoperoxidase labeling technique incorporating avidin–biotin–immunoperoxidase complex (Dako) was used. The sections were incubated with primary antibodies against mouse monoclonal NCAM (Dako) and mouse monoclonal 8-oxodG (Genox, Baltimore, MD) at room temperature. Separate sections were incubated with a relevant isotype-matched monoclonal antibody as a negative control. Endogenous peroxidase activity was quenched by adding 3% H2O2 for 7 minutes, followed by addition of a rabbit/mouse horseradish peroxidase solution for 30 minutes. Color was developed in solution containing diaminobenzidine tetrahydrochloride (Sigma), 0.5% H2O2 in PBS buffer (pH 7.6). Slides were counterstained with hematoxylin and mounted.

For NCAM and 8-oxodG expression on blood vessels, slides were analyzed by 3 blinded observers (MB, JO, and UF) using an established semiquantitative scoring method with a scale ranging from 0–4, where 0 = no staining, 1 = <25% staining, 2 = 25–50% staining, 3 = 50–75% staining, and 4 = >75% staining (4,5). NCAM staining was performed in a single run on the same day to control for peroxidase staining variability, and blood vessels were assessed for the percentage of staining and the strength (intensity) of staining in all vessels in the entire section (24). Then, in 5 hpf of tissue sections, we determined the percentage of individual blood vessels showing staining of NCAM in focal, discrete cells and the percentage showing NCAM staining as intact, continuous expression by adjacent cells. For 8-oxodG, blood vessels were assessed for cytoplasmic intensity, percentage of blood vessel with cytoplasmic staining, nuclear intensity, percentage of blood vessel with nuclear staining, and percentage of blood vessel stained for 8-oxodG in whole tissue, as previously described (24).

In vitro Matrigel assay of human dermal endothelial cell (HDEC) tube formation in response to hypoxia. Matrigel (Becton Dickinson, Franklin Lakes, NJ) basement membrane matrix was used to examine HDEC tube formation. Matrigel (50 μl) was plated in 96-well culture plate slides after thawing on ice and allowed to polymerize at 37°C in a 5% CO2 humidified atmosphere for 1 hour. HDECs were removed from culture, trypsinized, and resuspended at 4 × 104 cells/ml in full-strength EGM growth medium. Three hundred microliters of cell suspension was added to each chamber and incubated under either normoxic conditions or exposed to 3% hypoxia for 24 hours at 37°C in a 5% CO2 humidified atmosphere. EC tube formation was assessed using phase-contrast microscopy and photographed. A connecting branch between 2 discrete ECs was counted as 1 tube. The tube analysis was determined from 5 sequential fields (magnification ×40) focusing on the surface of the Matrigel.

Statistical analysis. SPSS, version 11 for Windows was used for statistical analysis. The nonparametric Wilcoxon signed rank test, Spearman’s correlation coefficient, and the Kruskal-Wallis test were used for analysis. P values less than 0.05 were considered significant. Results are expressed as the median and range unless specified otherwise.

RESULTS

Patient demographics. Forty-three subjects were recruited prior to starting therapy with biologic agents (21 patients with RA, 12 patients with PsA, 8 patients with OA, and 2 normal controls). Synovial tPO2 levels were recorded in 34 subjects. The median age of the cohort of patients with inflammatory arthritis (patients with RA and patients with PsA) was 52.37 years (range 27.26–80.22 years), and the clinical characteristics obtained for these patients included the tender joint count (median 5 [range 0–28]), swollen joint count (median 5 [range 0–19]), ESR (median 25 mm/hour [range 2–80 mm/hour]), CRP level (median 21 mg/liter [range 4–99 mg/liter]), DAS28 (median 4.11 [range 2.84–6.89]), global health, by VAS (median 46 mm [range 3–84 mm]), and disease duration (median 42 months [range 5–564 months]). Anti-CCP antibodies were found in 32.3% of these patients at a median titer of 15.6 (range
The OA patients in this cohort had a median age of 56.2 years (range 44–84 years) and a median disease duration of 12 months (range 1–192 months), and they were negative for rheumatoid factor and anti-CCP antibodies.

Immunofluorescent colocalization of FVIII and α-SMA in inflamed synovium. Representative images of dual staining for FVIII and α-SMA are shown in Figure 1. Sections from patients with inflammatory arthritis demonstrated a mixture of immature vessels, vessels

Figure 1. Representative separate and merged images of immunofluorescent factor VIII staining (red) and α-smooth muscle actin staining (green) in synovial tissue. **Top,** Varying degrees of blood vessel stability in rheumatoid arthritis synovium, including immature vessels (red; thin arrows), pericyte acquisition (red and green; thick arrow), and mature vessels (yellow; dashed arrow). **Middle,** Predominantly mature vessels in osteoarthritic tissue. **Bottom,** Predominantly mature vessels in normal tissue. (Magnification × 20.)

Figure 2. **A,** Immature blood vessels showing factor VIII (FVIII) staining (brown; thin arrow) and mature blood vessels showing both FVIII and α-smooth muscle actin (α-SMA) staining (brown and red; thick arrow) in rheumatoid arthritis synovium, double stained using the Dako EnVision G2 system. **B,** Representative images of serial sections stained for FVIII (top) and lymphatic vessel endothelial marker 1 (LYVE-1) (bottom), demonstrating that LYVE-1 and FVIII are expressed on different vessels. (Magnification × 20.) **C,** Number of blood vessels per high-power field (hpf) expressing FVIII (open bars) compared with the number coexpressing FVIII and α-SMA (shaded bars) in synovium from patients with inflammatory arthritis (IA), patients with osteoarthritis (OA), and normal controls. Boxes represent the 25th to 75th percentiles, lines within the boxes represent the median, and lines outside the boxes represent the 10th and 90th percentiles. * = P < 0.05.
acquiring pericytes, and stable vessels, which showed close alignment of ECs and pericytes. In OA and normal control tissue, all vessels had acquired pericytes and thus had undergone full maturation and stabilization. Immature and mature vessels were also identified and confirmed using immunohistochemistry (Figure 2A). In a subgroup of patients, we observed expression of FVIII independently of the lymphatic marker LYVE-1, which confirmed that the immature vessels were not lymphatics (Figure 2B). There was a significant difference between the number of vessels expressing FVIII and the number of vessels expressing FVIII/α-SMA in inflamed tissue ($P < 0.001$) (Figure 2C). In contrast, there was no significant difference between the number of blood vessels expressing FVIII and those expressing FVIII/α-SMA in OA or normal control tissue, suggesting vessel stabilization.

**Correlation of ST blood vessel expression of NCAM with disease activity.** NCAM expression was demonstrated in $25$–$50\%$ of vessels in the inflamed synovial joint. The expression pattern of NCAM varied in the inflamed ST (Figure 3A). Some blood vessels showed focal expression of NCAM, suggesting incomplete pericyte–EC interaction and unstable vessels, while others were entirely surrounded by NCAM-positive cells, suggesting a fully intact wall and mature vessels. NCAM expression was intact in OA tissue. The percentage of patients showing focal NCAM expression was significantly greater than the percentage showing intact NCAM expression, with $69\%$ of ST samples displaying focal expression and $31\%$ displaying nearly complete blood vessel expression ($P < 0.001$), suggesting that the majority of vessels remained unstable (Figure 3B). The number of NCAM-positive blood vessels in the entire section was significantly correlated with DAS28 ($r = 0.498, P = 0.013$), demonstrating a direct relationship between NCAM expression and disease severity. In a subgroup of patients, we performed NCAM and FVIII staining in serial sections to demonstrate colocalization of NCAM on FVIII-positive blood vessels. (Additional information is available from the corresponding author upon request.)

**Patterns of 8-oxodG expression in the inflamed ST.** Increased levels of oxidative DNA damage were demonstrated in the synovial joint tissue. Figure 3C shows representative images of immunohistochemical staining for 8-oxodG expression around blood vessels in the synovium. The pattern of 8-oxodG staining was both
nuclear and cytoplasmic, with minimal expression in OA tissue. Higher nuclear 8-oxodG expression was found compared with cytoplasmic staining (Figure 3D); however, this difference did not reach statistical significance. Figure 3C shows strong nuclear expression of 8-oxodG in blood vessels, in contrast to cells that accumulated in the lumen, which were negative for 8-oxodG, suggesting that oxidative damage is induced within the ST microenvironment.

**In vivo ST Po2, vascularity, and blood vessel stability.** We quantified Po2 in ST using the novel Licox probe, and found low levels of Po2 in the SM, with a median value of 22.8 mm Hg (range 3.2–54.1), consistent with median ambient oxygen tensions of 3.2% (range 0.45–7.7%). Figures 4A and B show representative images of macroscopic vascularity and microscopic vascularity expression in patients with low, medium, and high in vivo Po2 measurements (<10, 10–35, and >35 mm Hg, respectively), demonstrating an inverse relationship. At low Po2, there was an increase in macroscopic vascularity, with blood vessels demonstrating a tortuous pattern of vascular morphology (elongated with an increased diameter). At medium Po2 levels, an increase in blood vessels was also observed; however, vessels appeared more regularly shaped. Finally, in patients with high Po2 levels, a minimal number of blood vessels was observed.

At the microscopic level, low Po2 was associated with a significant increase in the number of blood vessels as compared with medium and high Po2 levels, where the number of vessels was significantly reduced (Figure 4B). When patients were grouped according to Po2 levels of <20 mm Hg versus >20 mm Hg, lower Po2 levels were significantly associated with increased numbers of vessels expressing FVIII (P < 0.04) and α-smooth muscle actin (α-SMA) (P < 0.03) (Figures 4C and D). There was no difference in the percentage of blood vessels coexpressing FVIII and α-SMA between patients with Po2 <20 mm Hg and those with Po2 >20 mm Hg, suggesting that low oxygen tension induces new vessel growth and acquisition of pericytes simultaneously.

Figures 5A and B show that low Po2 levels were associated with increased vessel NCAM expression compared with medium and high Po2 levels, where NCAM had a focal expression pattern. When NCAM levels were examined in patients with Po2 <20 mm Hg and those with Po2 >20 mm Hg, there was a significant increase in NCAM expression with low Po2 (Figure 5B). Figures 5C and D show 8-oxodG expression in samples from patients with high, medium, and low Po2 levels. Lower Po2 levels were associated with high 8-oxodG expression as compared with medium or high Po2 levels. When 8-oxodG levels were examined in patients with Po2 <20 mm Hg and those with Po2 >20 mm Hg, there was a significant increase in 8-oxodG expression with

![Figure 4](image_url)

**Figure 4.** A and B, Representative images of macroscopic vascularity (A) and microscopic vascularity (B) in samples from patients with low (left), medium (center), and high (right) in vivo tPo2 values. Levels of tPo2 were inversely associated with increased macroscopic vascularity and with the microscopic number of blood vessels. (Magnification × 20.) C and D, Number of blood vessels expressing factor VIII (C) and α-smooth muscle actin (α-SMA) (D) in patients with tPo2 <20 mm Hg and patients with tPo2 >20 mm Hg. Boxes represent the 25th to 75th percentiles, lines within the boxes represent the median, and lines outside the boxes represent the 10th and 90th percentiles. * = P < 0.05. BV = blood vessel.
low tPO₂ (Figure 5D), an effect which was significant when nuclear and cytoplasmic expression were analyzed separately (P < 0.01) (data not shown). Interestingly, no association between tPO₂ and sublining or lining layer expression of 8-oxodG was demonstrated. Expression of blood vessel 8-oxodG further contributes to the unstable environment.

Significant inverse correlations were also demonstrated between the in vivo tPO₂ level and macroscopic vascularity (r = −0.4, P = 0.004), between the tPO₂ level and NCAM expression (r = −0.44, P = 0.04), and between the tPO₂ level and nuclear and cytoplasmic expression of 8-oxodG (r = −0.42, P = 0.04 and r = −0.44, P = 0.03, respectively). There was no significant correlation between FVIII and NCAM expression, further confirming dysfunctional vessels. There was a significant inverse correlation of the tPO₂ level with the DAS28 (r = −0.439, P < 0.05) and the CRP level (r =

Figure 5. A and C, Representative images of NCAM expression (A) and microscopic vascular 8-oxodG expression (C) in samples from patients with low (left), medium (center), and high (right) in vivo tPO₂ values. Levels of tPO₂ were inversely associated with increased NCAM expression and increased 8-oxodG expression. (Magnification × 20.) B and D, NCAM (B) and 8-oxodG (D) expression in patients with tPO₂ < 20 mm Hg and patients with tPO₂ > 20 mm Hg. Boxes represent the 25th to 75th percentiles, lines inside the boxes represent the median, and lines outside the boxes represent the 10th and 90th percentiles. * = P < 0.05. See Figure 3 for definitions.

Figure 6. A and B, Human dermal endothelial cell tubule formation on Matrigel matrix following exposure to normoxia (A) and 3% hypoxia (B). Tube analysis was determined from 5 sequential fields focusing on the surface of the Matrigel. Arrows show connecting branches between endothelial cells (magnification × 40). C, Number of connecting branches per high-power field (hpf) following exposure to normoxia and 3% hypoxia. Bars show the mean and SEM of 5 experiments. * = P < 0.05.
−0.35, *P* < 0.05). Patients with tortuous blood vessel patterns had a significant increase in microscopic vascularity as compared with those with vessels with straight regular branching vessels (*P* < 0.05). Patients with tortuous blood vessel patterns also had a lower median ambient oxygen level of 2.6% (range 0.83–7%) compared with 3.3% (range 0.43–7.3%) in patients with straight regular branching vessels. This difference, however, was not significant.

**In vivo hypoxic levels induce in vitro angiogenesis.** Induction of HDEC tube formation on Matrigel matrices plated in 96-well culture plates was assessed under normoxic and 3% hypoxic conditions (reflecting the median level measured in vivo in the joint). Connecting branches and tube-like structures formed after 24 hours (Figures 6A and B). Cells incubated in 3% hypoxia showed a significant increase in tube formation. The mean ± SEM number of HDEC tubules formed increased from 9.8 ± 0.9 (normoxia) to 18 ± 1.4 (3% hypoxia; *P* < 0.05) (Figure 6C).

**DISCUSSION**

Angiogenesis is an early event and is highly dysregulated in inflammatory disorders, such as arthritis, psoriasis, and inflammatory bowel disease, and in many cancers. Activation and dysfunction of ECs and pericytes in these conditions are critically dependent on blood vessel maturity/stability and survival to preserve the integrity of newly formed vessels (6,7). This study is the first to show the presence of both immature and mature blood vessels in the inflamed synovium, with <50% of these vessels expressing NCAM, which was predominantly focal, suggesting that endothelial–pericyte interactions are not intact. In addition, this is the first study to demonstrate nuclear and cytoplasmic expression of 8-oxodG in the perivascular regions of the synovium. The evidence of DNA damage further supports the notion of an unstable vascular environment. Furthermore, we demonstrated that in vivo measures of tPO₂ in the inflamed joint were inversely correlated with macroscopic vascularity and microscopic blood vessel stability, NCAM expression, and DNA oxidative damage. Finally, HDECs exposed in vitro to Po₂ levels that reflected those measured in the joint showed a significant increase in angiogenic tube formation.

In this study, we demonstrated the presence of both immature and mature vessels in the inflamed synovium, with a significant proportion of vessels not having recruited supporting pericytes, which are essential for functional vascular patterning, diameter regulation, and vessel stabilization (12,30,31). This is in contrast to OA and normal ST, in which intact pericyte layers were observed. Furthermore, we demonstrated a direct relationship between low tPO₂ levels in the joint and macroscopic vascularity, microscopic vessel number, pericyte recruitment, and NCAM expression. This implies that in a hypoxic environment, synovial vessels are in a constant state of remodeling, and although many have recruited pericytes, vessels are still in an unstable state.

Pericyte recruitment and NCAM expression are critical for vessel stability in normal tissue. Acquisition of a pericyte layer ends the “plastic” state, thus stabilizing the vessel, as was demonstrated in OA and normal tissue in the present study. In pathologic states, pericyte coverage varies dramatically and is thought to be dysfunctional (32), with pericyte–EC contact constantly changing during the course of vessel development. This results in a deficiency in the ability of pericytes to mediate vascular stability. In cancer models, inhibition of VEGF results in regression of immature vessels, while vessels that are covered by pericytes remain (33), which is consistent with the results of studies showing that pericyte coverage is positively correlated with retinal vessel protection (34). Conversely, studies have also shown that retinal vessels abundantly covered by pericytes undergo regression when VEGF is suppressed. Therefore, the mere presence of pericytes on the vessel does not confer stability and thus protection against regression and may depend on EC–pericyte alignment through the regulation of ECM molecules, such as NCAM (35). NCAM deficiency results in blood vessel leakage, inhibition of pericyte recruitment, and continued vessel growth (13,14), an effect that may be mediated through altering the deposition of ECM molecules or by affecting PDGF-B (36,37).

Dysregulated angiogenesis is well recognized as one of the critical mechanisms involved in the progression of inflammation in arthritis. Previous studies have demonstrated increased angiogenesis and distinct vascular morphology in the SM in inflammatory arthritis (1,3,4). ECs lining synovial blood vessels express integrins, such as αvβ3, early in blood vessel formation, and later express adhesion molecules and Ki-67, which are markers of EC adhesion and proliferation (1,38,39). Activation, proliferation, and migration of ECs is fundamental for enabling activated monocytes to enter the SM via active recruitment, supporting the synovial pannus as it grows over the cartilage, eroding into bone, causing destruction and related disability (40). Dysfunctional vessels result in increased fenestration and leaki-
ness, abnormal morphology, and overexpression of specific molecules that sustain abnormal growth. Thus, synovial expression of EC αvβ3 integrin and many proangiogenic/antiangiogenic growth factors such as VEGF, Ang-2, Ang-1, PDGF, and TGFβ1 (4,5), coupled with the data from this study, suggests that synovial vessels are in a constant state of simultaneous formation, regression, and maturation.

This is a crucial finding in terms of implications for treatment, in particular the potential for clinical responsiveness in these patients. In vitro and in vivo cancer biology data have suggested that combination therapy targeting both the EC and pericyte may more efficiently inhibit blood vessel growth than therapy targeting either alone. Furthermore, for treatment to be most effective, synovial vessels should be targeted early in the disease process. This is consistent with previous studies demonstrating that synovial vascular development occurs before lining layer proliferation or leukocyte infiltration (41), as well as clinical studies demonstrating a window of opportunity in early arthritis during which therapy with biologic agents may be most effective (42).

We also demonstrated that low tPO2 levels were associated with increased macroscopic and microscopic vascularity. We observed a wide range of tPO2 levels within the joint that were inversely correlated with vascularity, vessel stability, and oxidative damage, further strengthening the hypothesis of an unstable synovial microenvironment. Furthermore, we found no association between NCAM expression and vascularity, suggesting that vessel stability is deficient. We showed that recapitulating the in vivo ST hypoxia levels in vitro leads to induction of angiogenic tube formation. Our data are consistent with previous morphometric studies showing that although there is an increase in blood vessel number, the expanding synovium increases at a greater rate, reflecting a greater metabolic turnover of the invasive pannus. Thus, the tissue remains hypoxic (43). Taken together, these data suggest that the neovascular network is dysfunctional in inflammatory arthritis, which may not only increase angiogenesis and vessel instability, but may also account for the persistence of inflammation.

In conclusion, these data demonstrate for the first time the presence of mature and immature vessels in the inflamed joint synovium. NCAM deficiency and oxidative DNA damage in a perivascular distribution suggests that pericyte acquisition may occur, many vessels remain in a plastic state. The relationship between tPO2 levels, increased vascularity, NCAM expression, and pericyte recruitment suggests that profound hypoxia may not only increase angiogenesis and vessel instability, but may also account for the persistence of inflammation.

**AUTHOR CONTRIBUTIONS**

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Fearon had full access to all of
the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Kennedy, Ng, Taylor, O’Sullivan, Veale, Fearon.

**Acquisition of data.** Kennedy, Ng, Biniecka, Saber, Veale, Fearon.

**Analysis and interpretation of data.** Kennedy, Ng, Biniecka, Taylor, O’Sullivan, Veale, Fearon.

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