Hypoxia, mitochondrial dysfunction and synovial invasiveness in rheumatoid arthritis

Ursula Fearon[1,2], Mary Canavan[2], Monika Biniecka[2] and Douglas J. Veale[2]

Abstract | Synovial proliferation, neovascularization and leukocyte extravasation transform the normally acellular synovium into an invasive tumour-like 'pannus'. The highly dysregulated architecture of the microvasculature creates a poor oxygen supply to the synovium, which, along with the increased metabolic turnover of the expanding synovial pannus, creates a hypoxic microenvironment. Abnormal cellular metabolism and mitochondrial dysfunction thus ensue and, in turn, through the increased production of reactive oxygen species, actively induce inflammation. When exposed to hypoxia in the inflamed joint, immune-inflammatory cells show adaptive survival reactions by activating key proinflammatory signalling pathways, including those mediated by hypoxia-inducible factor-1α (HIF-1α), nuclear factor κB (NF-κB), Janus kinase–signal transducer and activator of transcription (JAK–STAT) and Notch, which contribute to synovial invasiveness. The reprogramming of hypoxia-mediated pathways in synovial cells, such as fibroblasts, dendritic cells, macrophages and T cells, is implicated in the pathogenesis of rheumatoid arthritis and other inflammatory conditions, and might therefore provide an opportunity for therapeutic intervention.

The blood vessels of healthy, vascularized tissue are lined with a monolayer of quiescent endothelial cells organized as a 'phalanx' aligned in the direction of blood flow. As the blood vessels mature with an intact pericyte layer, the endothelial cells remain quiescent, and so minimal leukocyte migration occurs; in addition, the blood vessel is stable, and blood perfusion and the supply of oxygen are efficient. During inflammation, however, activated endothelial cells can lose their polarity, detach and protrude into the vessel lumen, thereby disrupting the pericyte layer. The resultant poorly organized vessel is dysfunctional; it increases stromal oedema and limits the delivery of nutrients and oxygen, causing hypoxia. Hypoxia is defined as inadequate oxygenation. Oxygen levels vary under physiological conditions, ranging from 150 mm Hg in the lung to 40–100 mm Hg in other organs[1]. Oxygen-sensing mechanisms have evolved to regulate endothelial cell and vessel morphogenesis to maximize perfusion and oxygen delivery to surrounding tissues[2]. Hypoxia has been implicated in the pathogenesis of rheumatoid arthritis (RA), as synovial hypoxia causes inflammatory cells to switch on invasive mechanisms, accelerate cell proliferation and enhance migration, and the levels of *in vivo* synovial oxygen correlate negatively with macroscopic synovitis and measures of disease activity[3–5]. Our knowledge of the partial pressure of oxygen (pO_2) in inflamed synovial tissue has, until a few years ago, been limited (BOX 1).

In the case of inflamed joints, activated endothelial vessels provide the gateway for leukocyte infiltration into the synovium (FIG. 1). The resultant increase in metabolic turnover of the expanding synovial pannus outpaces the dysfunctional oxygen supply, which, in turn, increases hypoxia and subsequent metabolic demand. Additionally, the increased distance between the vessels and the cellular stromal infiltrate increases the distance that molecular oxygen is required to diffuse[6]. Raised intra-articular pressures owing to movement and joint swelling caused by inflammation might further compromise the vascular supply to the inflamed joint, thereby exacerbating the hypoxic environment.

The complementary actions of vascular endothelial growth factor (VEGF) and members of the angiopoietin (Ang) family are critical for the maintenance of vessel stability, vascularization and regression during the formation of the RA vasculature[7–9]. The expression of Ang1, Ang2 and their receptor Tie2 is significantly increased in whole paws during disease progression in collagen-induced arthritis (CIA) mouse models, and the blockade of Tie2 ameliorates bone destruction[10,11]; Tie2 has also

[1]*The Department of Molecular Rheumatology, Trinity College Dublin, The University of Dublin, College Green, Dublin 2, Ireland.*
[2]*The Centre for Arthritis and Rheumatic Disease, Dublin Academic Medical Centre, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland.*
Correspondence to D.J.V.
douglas.veale@ucd.ie

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Key points

- Hypoxia, arising as a consequence of the increased cellular demand for oxygen during the inflammatory response, is a powerful trigger for the activation, proliferation and survival of endothelial cells and fibroblast-like synoviocytes
- Impaired mitochondrial function and oxidative damage caused by hypoxia further exacerbate the inflammatory response through metabolic perturbation
- Hypoxia induces immune cell dysfunction, resulting in an altered metabolic profile
- The hypoxic environment induces activation of a complex crosstalk of signalling pathways, providing a feedback loop leading to further activation and inflammation
- Targeting synovial metabolic pathways through inhibition of hypoxia-induced signalling pathways might have therapeutic benefit for rheumatoid arthritis and other inflammatory diseases

been shown to mediate angiogenesis induced by Toll-like receptor 2 (TLR2) in RA[12]. Platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) also regulate vessel stability and can induce the invasion of fibroblast-like synoviocytes (FLSs)[13,14]. Unstable vessels in the inflamed joint are associated with the presence of incomplete interactions between endothelial cells and pericytes, hypoxia and increased oxidative damage[4,15] (FIG. 1). Oxidative damage, which arises in RA synovial tissue through the detrimental effects of hypoxia on mitochondria, might mediate the activation of endothelial cells as well as promoting angiogenesis and cartilage damage by upregulating matrix degradation[16,17]. Oxidative damage might also induce the expression of adhesion molecules that influence both vessel stability and leukocyte migration.

In this Review, we examine the emerging evidence for the role of hypoxia in immune-inflammatory responses in RA, focusing on mitochondrial dysfunction, the activation of proinflammatory signalling pathways and the reprogramming of metabolic activity. Understanding the regulation of hypoxia and metabolic perturbation in inflammation, and resolution, might provide a basis for novel therapies.

Box 1 | **Measuring hypoxia in the rheumatoid joint**

Originally, studies used surrogate markers to measure hypoxia in the synovial fluid of patients with rheumatoid arthritis (RA)[54,150]. Then, in 1970, Lund-Olesen used a Clark-type electrode to demonstrate significantly lower partial pressure of oxygen (pO_2) levels in the synovial fluid of patients with RA compared to those with osteoarthritis[151]. Low oxygen levels have also been reported in the tenosynovium of RA patients undergoing repair surgery[152]. In 2008, we developed a method to directly measure pO_2 in synovial tissue using a 2.7 mm needle arthroscope (Wolf, Illinois) and a LICOX® combined pO_2 and temperature probe (CC1.P1, Integra Life Sciences Corporation, New Jersey, USA). Oxygen that diffuses from the tissue through the polyethylene wall into the inner electrolyte chamber of the probe is transformed to OH^- ions at a negatively charged polarized precious metal electrode, the polarographic cathode. In this way, following synovial biopsy under direct visualization, the LICOX® probe registers a stable *in vivo* measurement of pO_2 and temperature in the synovial tissue[3]. The measurements are taken every 5 min for approximately 20 min until a steady state is achieved. This method, which has been extensively validated, demonstrates profound hypoxia in inflamed synovial joints (median synovial oxygen levels 3.2% [range 0.46–7%]).

Hypoxia and mitochondrial damage

Mitochondria carry out a central role in the regulation of cellular bioenergetics and metabolism, and facilitate cellular stress responses. Hypoxia induces a wide spectrum of alterations in mitochondrial structure, dynamics and genome stability, resulting in reduced mitochondrial respiration, excessive production of reactive oxygen species (ROS), loss of ATP, increased oxidative damage and the accumulation of mitochondrial (mt)DNA mutations[18,19]. Damaged mitochondria also release molecules that can translocate outside the organelle and promote immune responses[20].

ROS, oxidative stress and DNA damage

ROS, oxidative stress and mitochondrial alterations have been implicated in the pathogenesis of joint inflammation[21]. ROS stimulate FLSs to secrete matrix metalloproteinases, inhibit the synthesis of cartilage proteoglycans and accelerate bone resorption[16,22]. Higher levels of oxidative DNA damage, as measured by 8-oxo-dG, were detected in mononuclear cells and granulocytes, as well as in serum, synovial fluid and urine, from arthritic patients when compared with healthy controls[23–25], and the presence of vascular 8-oxo-dG is associated with synovial immature vessel status and with low levels of synovial oxygen[4]. Similarly, levels of lipid peroxidation are high in the inflamed joint and correlate inversely with synovial pO_2 levels, which reflects mitochondrial damage within the inflamed joint[26].

Mitochondrial dysfunction in normal synoviocytes induces the expression of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) and IL-8, and amplifies the responsiveness to cytokine-induced chondrocyte inflammation through the production of ROS and activation of nuclear factor κB (NF-κB)[27,28]. Similarly, ROS induce the expression of COX-2 in FLSs through phosphorylation of mitogen-activated protein kinases and NF-κB, providing a functional link between mitochondrial dysfunction and synovial cell activation[29]. The immediate-early response gene X-1 (*IEX-1*, also known as *IER3*) is involved in preventing the production of ROS in mitochondria and, consequently, null mutation of *IER3* increases the production of mitochondrial ROS. This increase subsequently facilitates the differentiation of T helper 17 (T_H17) cells, the increased production of IL-17, and more severe arthritis in Ier3 null mice than in wild-type mice after immunization with collagen, indicating that mitochondrial alterations contribute substantially to the dominant T_H cell effector phenotype[30].

The mitochondrial genome is highly susceptible to mutagenesis, and elevated oxidative stress contributes to somatic mtDNA mutations. We examined synovial tissue from patients with RA for the presence of mtDNA mutations and demonstrated an increase in mitochondrial mutagenesis associated with lower pO_2 in the synovium, suggesting that the accumulation of random mitochondrial mutations is driven by the hypoxia-induced overproduction of ROS[31]. The mutations detected were mainly transitions, which are characteristic of oxidative stress[32]. The accumulation of random synovial mitochondrial mutations in response

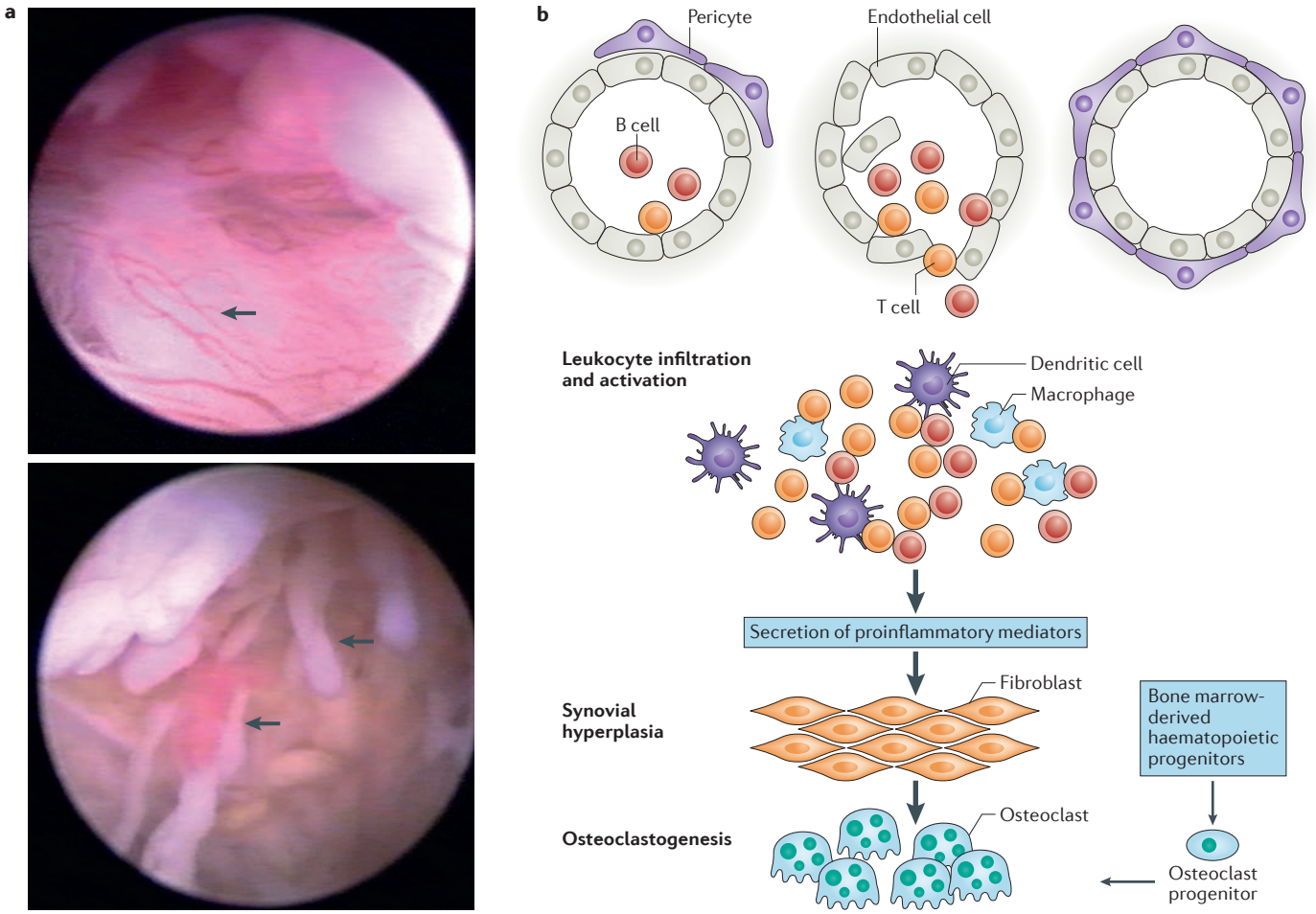

Figure 1 | **Blood vessel activation in the rheumatoid joint. a** | Direct visualization by arthroscopy of a joint affected by inflammatory arthritis reveals a swollen, oedematous synovial lining tissue that is red from angiogenesis (arrow, upper panel) and hyperaemia and often forms villi (arrows, lower panel). **b** | Within an inflamed rheumatoid joint, a mixture of immature and mature blood vessels are present, suggesting blood vessels are probably undergoing simultaneous angiogenesis, pericyte recruitment and stabilization. The activation of immature synovial vessels facilitates the recruitment and migration of immune cells into the synovial membrane. These cells release proinflammatory mediators, which activate fibroblast-like synoviocytes, resulting in synovial hyperplasia, which, in turn, leads to osteoclastogenesis, bone resorption and, ultimately, joint destruction.

to hypoxia suggests the promotion of a mitochondrial mutator phenotype that might be involved in regulating inflammatory responses. This notion is consistent with previous studies demonstrating increased DNA damage and dysfunctional mitochondria in synovial tissue and peripheral lymphocytes from patients with RA[4,33]. An increased frequency of clonal mtDNA mutation in *MT-ND1*, which encodes mitochondrial NADH dehydrogenase-1, has been detected in RA FLSs, with potential mutation sites in the MHC epitope in patients with RA, but not those with osteoarthritis. This increase in somatic mutations might influence cellular function by contributing to the mechanisms involved in the transformed phenotype of RA FLSs, which, along with their association with the MHC epitope, might further perpetuate the immune response[34]. Furthermore, synovial tissue pO_2 levels were reported to increase in patients who responded to biologic therapy, leading to a less hypoxic microenvironment and a significant decrease in mtDNA mutations and disease activity[35]. Fibroblasts from mice that lack TNF receptor (TNFR)1 have been described as having altered mitochondrial function that results in enhanced oxidative capacity as well as the increased generation of mitochondrial ROS and proinflammatory cytokines[36]. Moreover, using RA FLSs, we have shown *in vitro* that the stability of mtDNA and mitochondrial function are altered by the presence of TNF or hypoxia, thus recapitulating the *in vivo* responses[37].

Mitochondrial DAMPs

Inflammation is characterized by elevated levels of damage-associated molecular pattern (DAMP) molecules, which are released as a result of tissue injury. The released mitochondrial DAMPs can induce innate or adaptive immune responses by activating cell surface receptors (such as the P2X purinoceptor 7 (P2X7R) or *N*-formyl

peptide receptors (FPRs)). Damaged mitochondria have been reported to contain at least two unique molecular signatures, including free mtDNA and *N*-formyl peptides, which are known to contribute to proinflammatory responses (FIG. 2a). Furthermore, Nod-like receptors (NLRs) comprise a large family of intracellular proteins that are involved in the innate immune responses to microbial pathogens through the recognition of conserved pathogen-associated molecular patterns. The NLRP3 inflammasome is very well studied. Upon inflammasome activation, caspase-1 controls the activation and cellular release of active IL-1β and IL-18, both of which have been implicated in RA. Dysfunctional mitochondria have been implicated in the activation of the NLRP3 inflammasome through the generation of mitochondrial ROS and the release of mtDNA.

Free mtDNA. Mitochondrial DNA contains inflammatogenic unmethylated CpG DNA repeats, which function as ligands for TLR9, a member of the highly conserved pattern-recognition receptors (PRRs)[20]. Bacterial DNA containing unmethylated CpG motifs has been shown to induce arthritis in mice[38], as has endogenously oxidized mtDNA, which displays immunostimulatory properties *in vitro* and *in vivo* owing to the presence of unmethylated CpG sequences[39]. Not only have significantly higher levels of extracellular mtDNA been detected in the plasma and synovial fluid of patients with RA compared with non-arthritic controls, but a strong correlation between the levels of both extracellular mtDNA and 8-oxo-dG and the presence of rheumatoid factor has been shown in the synovial fluid of patients with RA[25]. Accordingly, a vicious cycle between mitochondrial dysfunction and inflammation exists, in which cell-free mtDNA activates phagocytes to produce tissue-destructive enzymes and proinflammatory cytokines, which contribute to inflammation and thereby accelerate the release of additional endogenous inflammatory mtDNA. mtDNA induces inflammation and lung injury in rats and increases the expression of TLR9 and NF-κB in both rat lung tissue and macrophage cultures derived from rat peritoneum[40]. Treatment of plasmacytoid dendritic cells (DCs) with either native mtDNA (containing unmethylated CpG DNA repeats) or oxidatively modified mtDNA upregulated the cell-surface expression of a co-stimulatory molecule (CD86), maturation marker (CD83) and antigen-presenting molecule (HLA-DQ), as well as increasing the production of TNF and IL-8 (REF. 41).

N-formyl peptides. Mitochondria use an *N*-formyl-methionyl-tRNA as an initiator of protein synthesis, and *N*-formyl peptides constitute another molecular signature of damaged mitochondria. *N*-formyl peptide sequences, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLF), are potent chemoattractants. fMLF activates neutrophil chemotaxis and mediates antimicrobial responses by binding to FPRs and, whereas knockout of FPR1 and FPR2 increased the severity of infection, FPR agonists inhibited the secretion of proinflammatory cytokines after microbial infection in mice[42]. In a K/B×N mouse model of RA, an FPR agonist significantly reduced clinical disease severity and attenuated osteoclastogenesis[43].

The NLRP3 inflammasome and autophagy. Cytosolic mtDNA released from damaged mitochondria can activate the NLRP3 inflammasome, leading to the subsequent activation of caspase-1 and secretion of IL-1β and IL-18 (REF. 44). NLRP3 is also activated by ATP, which is currently considered not only as a mitochondrially produced intracellular energy source, but also as an important extracellular signalling molecule. Extracellular ATP promotes neutrophil chemotaxis through the release of CXCL8, the adhesion of neutrophils to endothelial cells, and the secretion of IL-1β and IL-18 through NLRP3 inflammasome activation in macrophages[45,46]. Furthermore, mitochondrial ROS have the ability to induce NLRP3 inflammasome activation. However, although NLRP3 is expressed in RA synovium, to date no study has demonstrated redox activation of inflammasome components in RA.

Autophagy is a process of lysosome-mediated degradation of organelles and proteins that is activated by conditions of cellular stress, including hypoxia, ROS, endoplasmic reticulum (ER) stress and microbial infection. A dual role for autophagy in the regulation of cell death pathways has been demonstrated in RA FLSs: autophagy promoted ER-stress-induced cell death but also protected against apoptosis induced by proteasome inhibition. Furthermore, autophagy and resistance to apoptosis were more pronounced in methotrexate-treated FLSs from patients with RA compared with synovial fibroblasts from patients with osteoarthritis. Inhibition of autophagy in RA FLSs increased their susceptibility to methotrexate, inducing cell death, thereby confirming an apoptosis-resistant phenotype of RA FLSs[47,48]. Defective mitochondria that overproduce ROS are eliminated by autophagy to restore redox homeostasis. In a study by Zhou *et al.*[49], pharmacologic or genetic inhibition of autophagy caused the accumulation of dysfunctional ROS-generating mitochondria, which was accompanied by spontaneous activation of the NLRP3 inflammasome and the release of IL-1β. Nakahira *et al.*[44] propose that autophagic proteins are important for the regulation of inflammasome-dependent inflammation, as depletion of autophagic proteins promoted the accumulation of dysfunctional mitochondria, cytosolic translocation of mtDNA, activation of caspase-1 and the secretion of IL-1β and IL-18.

Hypoxia and metabolism

The increased proliferation and rapid activation of immune cells during inflammation causes them to undergo a metabolic switch in favour of glycolysis over oxidative phosphorylation. This metabolic shift, which enables energy to be produced independently of the oxygen supply, occurs in many hypoxia-associated inflammatory conditions, including RA[50]. The activation of the transcription factor hypoxia-inducible factor (HIF)-1α in response to low pO_2 (discussed below) modulates the activity of a number of genes and, by inducing genes that encode glucose transporters and glycolytic enzymes, HIF can promote the production of glycolytic energy (FIG. 2b). Studies have shown an increase in the levels of the glucose transporter GLUT1

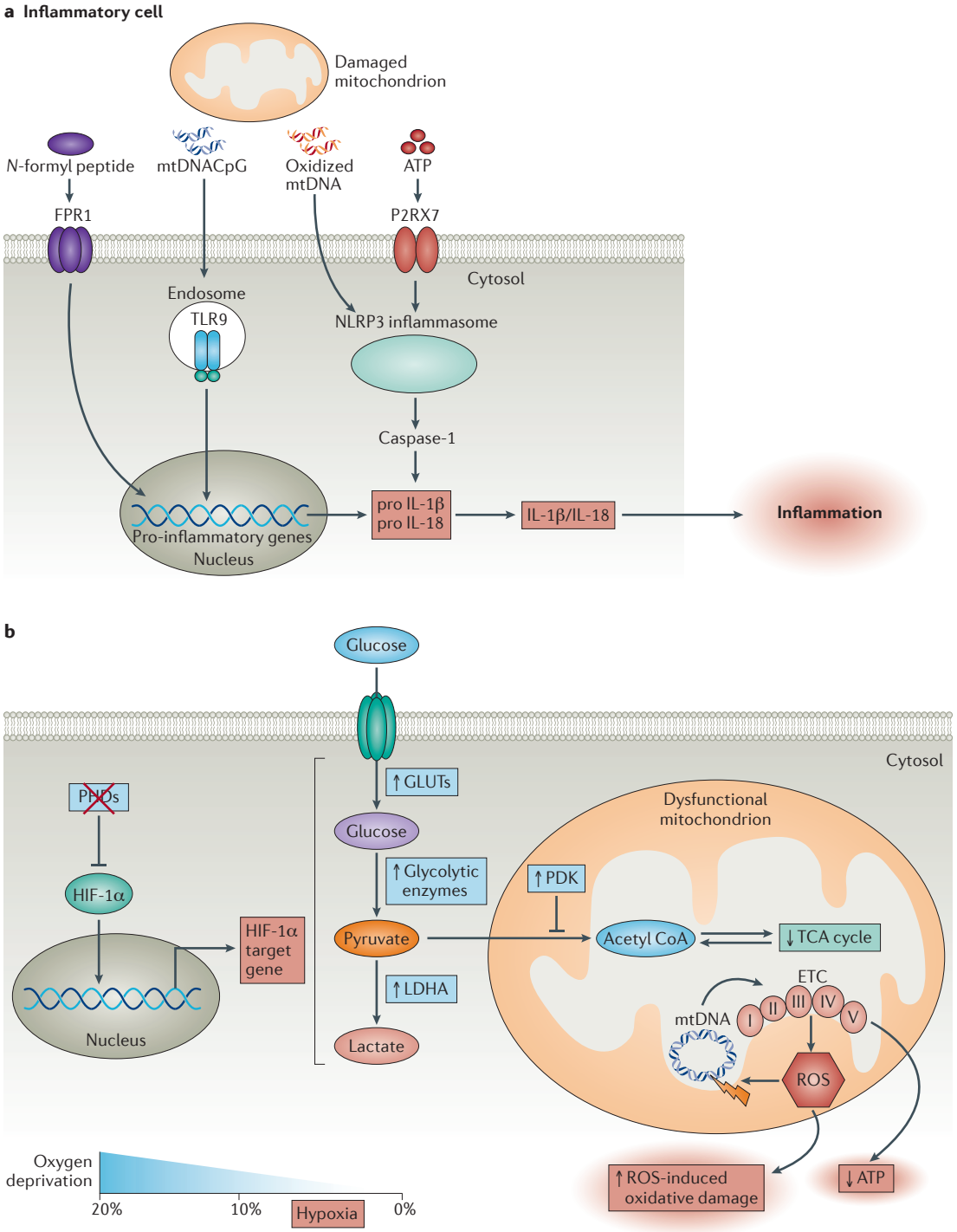

Figure 2 | **Hypoxia and mitochondrial dysfunction. a** | Cellular injury and necrosis trigger the release of mitochondrial damage-associated molecular patterns (DAMPs), such as *N*-formyl peptides, mitochondrial (mt)DNA and ATP, from damaged mitochondria, which are potent immunological activators. The released mtDAMPs can initiate innate or adaptive immune responses by activating cell surface receptors (such as the P2X7 receptor P2X7R, or formyl peptide receptors) or intracellular receptors (such as Toll-like receptor 9 (TLR9) or NLRP3). **b** | Reduced oxygen conditions stabilize hypoxia-inducible factor (HIF)-1, which targets genes involved in hypoxic cell metabolism. HIF-1 promotes the activation of glycolysis by upregulating the expression of glucose transporters (GLUTs), therefore increasing glucose uptake into the cell; intracellular glucose is metabolized by the HIF-dependent increase of the glycolytic enzymes. Elevated glycolysis generates pyruvate, which is largely converted to lactate by HIF-inducible lactate dehydrogenase A (LDHA). HIF-1 also induces pyruvate dehydrogenase kinase (PDK), diminishing pyruvate entry into the tricarboxylic acid (TCA) cycle and subsequently decreasing mitochondrial oxidative phosphorylation. Hypoxia increases the production of reactive oxygen species (ROS), the primary source of mitochondrial genomic instability, leading to respiratory chain dysfunction. Excess ROS cause oxidative damage to DNA, lipids, proteins, altering cell functions. ETC, electron transport chain.

in articular chondrocytes in response to hypoxia[51] and in glyceraldehyde 6-phosphate dehydrogenase in cartilage explants exposed to oxidative damage[52]. Pyruvate dehydrogenase kinase (PDK) is an enzyme that inactivates pyruvate dehydrogenase resulting in the conversion of pyruvate to acetyl co-A which is then oxidized through the Krebs cycle to produce energy.

HIF-1α also increases the activity of lactate dehydrogenase A (LDHA), which converts pyruvate to lactate; the resultant acidic environment promotes cell proliferation, invasiveness and mutations. In patients with RA, increased activity of lactate dehydrogenase was demonstrated in the synovium[53], elevated lactate levels correlated with diminished levels of glucose in synovial fluids[54,55] and the degree of synovial lactic acidosis was associated with the level of synovial inflammation[56]. Increased acidosis in tissues/cells is known to cause mutations, resulting in defects in DNA repair mechanisms, which can lead to transformation of normal cells and prevent apoptosis. The switch to glycolysis in the inflamed joint and the acidic microenvironment might contribute to the transformed phenotype of RA FLSs and to altered expression of p53 tumour suppressor gene, which is known to be dysregulated in the RA synovium[57,58]. In turn, this altered expression might inhibit DNA repair, thus interfering with apoptotic mechanisms and promoting cell survival[57,58]. In human and mouse macrophages and an *in vivo* mouse model of atherosclerosis, hypoxia potentiated the glycolytic flux induced by upregulation of proinflammatory activity in a manner that was dependent on both HIF-1α and 6-phosphofructo-2-kinase[59]. Furthermore, high concentrations of glucose increased the secretion of IL-1β from RA monocytes through an NLRP3-dependent mechanism[60].

The effect of hypoxia on immune cells

As immune cells develop, differentiate and migrate to different tissues, they encounter fluctuations in oxygen tension; accordingly, they have developed the ability to adapt to this adverse environment.

Hypoxia and T cells

Low pO_2 can promote the survival of T cells by stabilizing HIF-1α[61]. Gaber *et al.* showed that the expression of HIF-1α was increased in the synovium of patients with RA and that $CD4^+$ T cells adapt to hypoxic conditions through activation of HIF-1α-driven pathways[62]. Hypoxia can also further enhance the stabilization of HIF-1α that is induced by T-cell-receptor-mediated activation of the phosphatidylinositol 3-kinase–mechanistic target of rapamycin (PI3K–mTOR) pathway[63]. Although HIF-1α activation has been identified in T cells at sites of inflammation and is thought to facilitate the ability of T cells to maintain immunity in hypoxic tissues, evidence has emerged that HIF-$1α^+$ T cells also contribute to the progression of inflammatory disease, and several T-cell subsets with distinct functions in immunity and inflammation have been identified: T_H1, T_H2, T_H17, T_{REG}, T_H9 and T_{FH} cells[64–67]. These subsets retain a level of plasticity and are not terminally differentiated. $FOXP3^+$ regulatory T cells (T_{REG} cells) have been shown to convert to pathogenic T_H17 cells in the context of RA[68], which might, in part, be driven by the hypoxic microenvironment of the joint. Moran *et al.*[69] demonstrated that patients with RA with lower pO_2 levels in synovial tissue had significantly higher numbers of IL-$17A^+$ mononuclear cells than patients with higher pO_2 levels. Dang *et al.*[70] reported that HIF-1α could induce the transcription of retinoic acid-receptor-related orphan receptor-γt (RORγt) while simultaneously targeting FOXP3 for degradation, thereby promoting the generation of T_H17 cells over T_{REG} cells.

Other studies have also highlighted the role of HIF signalling in mediating T_H17–T_{REG} cell-fate decisions, providing evidence that hypoxia can alter T-cell lineage decisions[71]. $CD161^+$ T_H17 cells that were resistant to suppression by T_{REG} cells, polyfunctional in their cytokine production, highly proliferative and capable of activating FLSs were identified in the hypoxic RA joint[72]. Conversely, hypoxia can also control T_{REG} cell development through induction of FOXP3 in models of intestinal inflammation[73]. It is therefore likely that oxygen gradients and the cytokine milieu at the site of inflammation might together determine T-cell differentiation.

The hypoxic environment itself can also induce metabolites and growth factors which, in turn, can affect T-cell function. Lactic acid enhances the secretion by human monocytes and macrophages of IL-6 and IL-23, which are required to maintain T_H17 cells[74]. Lactic acid has also been shown to inhibit T-cell motility and enhance IL-17 production[75].

Hypoxia and macrophages

Macrophages are found in abundance within the inflamed synovium[76]. The ability of monocytes to traffic from blood to inflammatory tissues is mediated by chemokines and possibly hypoxic gradients[77]. Hypoxia induces the translocation of HIF-1α and HIF-2α into the nucleus in isolated macrophages[78], and the expression of matrix metalloproteinase-7 (MMP-7), neuromedin B receptor and DNA-binding protein inhibitor (Id2), as well as known hypoxia-inducible genes such as *VEGF* and *GLUT1* (REF. 79). Furthermore, numerous studies have shown that hypoxia can alter the morphology, survival, phagocytosis, metabolic activity, cytokine secretion and cell-surface protein expression by macrophages[80], which, in turn, can induce neovascularization and further activation of immune cell responses, thus perpetuating the inflammatory response.

Conditional-knockout experiments in mice indicated that HIF-1α is essential for the inflammatory activity of myeloid cells. Targeted deletion of *Hif1a* in myeloid cells in arthritic mice reduced macrophage mobility, invasiveness and bactericidal activity, resulting in reduced synovial infiltration of macrophages and a marked reduction in joint swelling[77]. The expression of $CD68^+$ macrophages in synovial tissue is inversely related to *in vivo* synovial pO_2 levels and correlates with disease activity; these effects are reversed in patients who respond to treatment with TNF inhibitors[3,5]. Unsurprisingly, TNF in combination with low pO_2 levels can further enhance the survival of macrophages[81].

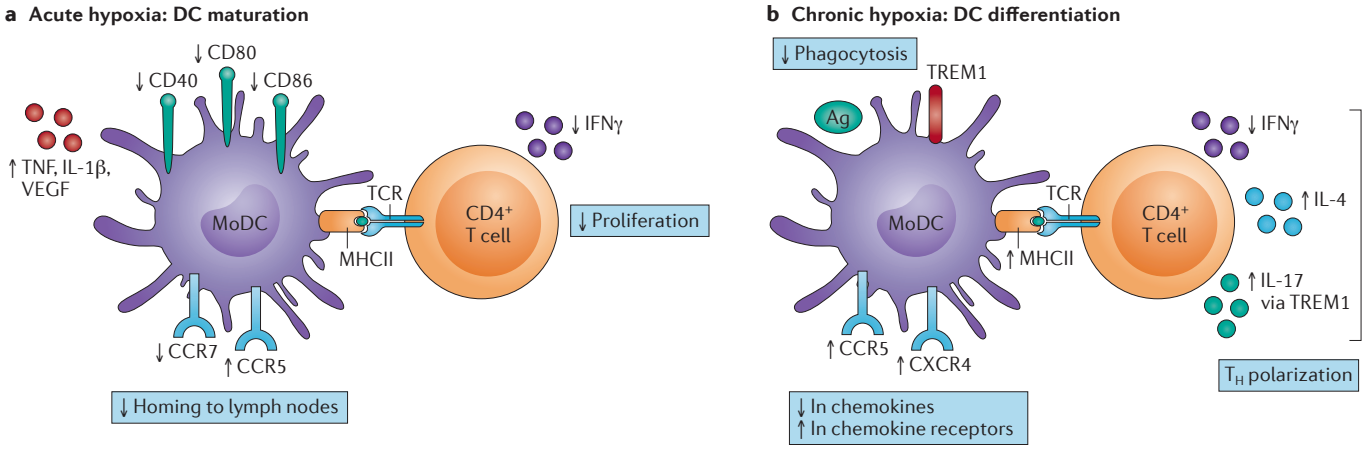

Figure 3 | **Acute and chronic hypoxia regulates dendritic cell function. a** | Monocyte-derived dendritic cells (MoDCs) differentiated under normoxia and subsequently activated or matured under acute hypoxia display altered inflammatory capabilities as observed by decreased expression of the co-stimulatory markers CD86, CD80 and CD40, increased production of TNF, IL-1β and vascular endothelial growth factor (VEGF), and a decreased ability to induce T-cell proliferation. MoDCs matured under acute hypoxia also have altered chemotactic responses, promoting cell migration to inflamed tissues in addition to impairing cellular homing to lymph nodes. **b** | Exposure of monocytes to hypoxia during their differentiation into DCs (chronic hypoxia) can decrease phagocytosis and antigen (Ag) uptake. Some reports suggest these cells have decreased expression of maturation markers whereas other groups have reported no change in these markers. These cells also express the hypoxia-inducible marker triggering receptor expressed on myeloid cells 1 (TREM1), which, upon ligation, promotes the differentiation of T_H17 cells. In co-culture models, T cells cultured with hypoxia-differentiated MoDCs secrete lower levels of IFNγ but increased levels of IL-4. These cells have high sensitivity to chemoattractants, ensuring they are ready to leave hypoxic tissue, migrate to lymph nodes and initiate adaptive immune responses. DCs express a wide variety of chemokine receptors (CCR2, CCR5, CCR7 and CXCR4) that respond to their respective chemokine ligands (MCP-1, RANTES, CXCL19 and CXCL12, respectively) in order to facilitate specialised migration. In order for DCs to effectively function, they utilise these chemotactic properties to traffic to inflamed sites within the body and subsequently migrate as mature DCs to the draining lymph node to induce adaptive immune responses. TCR, T-cell receptor.

In myeloid cells, HIF-1α increases the transcription of glycolytic enzymes, thereby increasing glucose uptake and the glycolytic rate, consequently limiting oxidative phosphorylation[77]. As M1 and M2 polarized macrophages have been shown to preferentially use different metabolic pathways, with M1 relying on glycolysis and M2 relying on oxidative phosphorylation for energy, these results suggest that hypoxia might drive an M1 macrophage phenotype[82]. Furthermore, studies have demonstrated that different signalling mechanisms can occur in monocytes and macrophages as they adapt to a hypoxic environment: whereas HIF-1α activation predominates in macrophages, monocytes use NF-κB1 to regulate the expression of hypoxia-induced genes[78].

Hypoxia and dendritic cells

During the differentiation of monocytes into immature DCs under hypoxic conditions *in vitro*, the expression of over 2,000 genes was reported to be induced[83]. Among these genes were those involved in glycolysis, the pentose phosphate pathway, antigen processing and presentation pathways, and cell migration[83]. DCs that mature under conditions of acute hypoxia show a decreased migratory capacity and increased inflammatory capabilities[84], whereas those that mature under chronic hypoxia develop a migratory phenotype through the modulation of genes that encode chemokines and chemokine receptors, and those that are involved in cell adhesion and tissue remodelling[83–86] (FIG. 3). Mature DCs express an array of cell-surface receptors, including CD86, CD80 and CD40, that induce T-cell proliferation and activation via co-stimulation. These T cells can then produce a variety of proinflammatory and anti-inflammatory cytokines such as TNF, IFNγ, IL-17 and IL-4. Mancino *et al.*[84] demonstrated that acute hypoxia upregulates the expression of *CCR5*, which encodes a protein involved in the responsiveness of DCs to inflammatory chemokines. Moreover, hypoxic DCs injected into the footpads of wild-type mice showed defective homing to draining lymph nodes, whereas leukocyte recruitment to the site of injection was enhanced[84], suggesting that exposure of DCs to hypoxia promotes a dissociation between their inflammatory and tissue repair functions, which has implications for tissue homeostasis. By contrast, chronic hypoxia can induce chemotactic responses via upregulation of chemokine receptors and increases in ligand responsiveness, while simultaneously inhibiting the production of inflammatory chemokines[87]. Yang *et al.*[88] reported decreased maturation, migration and antigen uptake in DCs in the presence of hypoxia. Furthermore, studies have also emerged highlighting the effect of hypoxia-treated DCs on T-cell differentiation, with implications for cell migration and activation in tumours and at sites of inflammation[88,89]. Finally, the expression

of *TREM1*, a hypoxia-inducible gene that encodes a protein involved in amplifying the immune response, has been detected on DCs isolated from the synovial fluid of patients with juvenile idiopathic arthritis[85].

Hypoxia signalling pathways in RA

Hypoxia-inducible factor

As the master regulator of oxygen homeostasis, HIF executes the cellular response to altered levels of oxygen[90]. HIF is a heterodimeric transcription factor composed of two subunits: HIF-1α, the expression of which is regulated by oxygen availability, and HIF-1β, which is constitutively expressed in the cell nucleus. HIF-1α senses oxygen through the activity of the oxygen-dependent hydroxylase enzymes prolyl hydroxylases 1–3 (PHD1–3) and asparagine hydroxylase factor inhibiting HIF (FIH)[91]. Under normoxic conditions, the prolyl hydroxylases hydroxylate two prolyl residues on HIF-1α (pro402 and pro564), which generates a binding site for the von Hippel–Lindau tumour suppressor protein (VHL)[92]. VHL is an E3 ubiquitin ligase, and subsequent polyubiquitylation of HIF targets it for proteasomal degradation. Under hypoxic conditions, however, hydroxylase activity is inhibited, so HIF-1α subunits accumulate; they translocate to the nucleus where they dimerize with HIF-1β and its cofactor p300/CBP. This HIF-1α complex binds to specific DNA motifs and regulates the transcription of hundreds of genes, each containing hypoxia-responsive elements (HREs) (FIG. 4), involved in survival, metabolism, angiogenesis and invasion.

Studies have shown that the expression of HIF-1α and HIF-2α is increased in the RA synovium, in both the lining layer and sublining, and that this increase is associated with increased synovial vascularization and inflammation[93,94]. HIF-1α overexpression enhances the expansion of inflammatory T_H1 and T_H17 cells mediated by RA FLSs, resulting in the increased production of IFNγ and IL-17 (REFS 95,96). IL-17 and TNF synergistically induce a HIF-1α-dependent invasive phenotype in RA FLSs[97]. Hypoxia potentiates the effects of IL-17A, IL-1β and TNF on angiogenic and invasive mechanisms in RA through the activation of HIF-1α, as well as NF-κB[98,99]. Hypoxia, through HIF-1α, has also been seen to potentiate the TLR-induced expression of cytokines, metalloproteinases and VEGF[95].

In animal models of arthritis, HIF-1α expression has been detected in the synovium as early as 1 week after collagen injection, before clinical evidence of arthritis, suggesting that HIF-1α responses are involved in RA pathogenesis at a very early stage of disease[100]. Decreased infiltration of myeloid cells to the joint, reduced paw swelling and decreased disease development were observed in CIA models using HIF-1α-deficient macrophages[94]. FLSs enhance angiogenesis and the subsequent recruitment of myeloid cells through the activation of HIF-1α in immunodeficient mice[101]. Pretreatment of mice with chronic intermittent hypobaric hypoxia confers a protective effect against CIA through the downregulation of HIF-1α and inhibition of both TNF and IL-17 and the ratio of T_H1/T_H2 lymphocytes[102]. Finally, amino-terminal mutation of the FOXP3 transcription factor in T_{REG} cells blocks interactions with HIF-1α but increases those with interferon regulatory factor (IRF)4, which confers protection against antibody-mediated arthritis in the K/B×N model[103]. In summary, HIF-1α expression is increased in the synovium of patients with RA, where it is associated with markers of inflammation and disease activity. In synovial cells and animal models of arthritis, overexpression of HIF-1α signalling increases angiogenesis, activation of immune cells, secretion of proinflammatory mediators and invasive mechanisms, whereas blockade of HIF-1α promotes resolution of inflammation.

Prolyl hydroxylases

Although prolyl hydroxylases regulate HIF activation and stability, little is known about their expression and regulation in the inflamed joint. Transient silencing of these enzymes in fibroblasts not surprisingly induces the expression of many pro-angiogenic and inflammatory mediators by increasing the levels of HIF[104]. Muz *et al.*[105] demonstrated that PHD2 was the prominent prolyl hydroxylase in RA FLSs, and that its knockdown augmented HIF-1α-induced gene expression. Similarly, upregulation of miR-210 by connective tissue growth factor through PI3K–AKT and NF-κB pathways repressed the activity of prolyl hydroxylases and subsequently promoted HIF-1α-dependent VEGF expression in synoviocytes from patients with osteoarthritis[106]. Treatment of FLSs with dimethyloxalylglycine, a pan-hydroxylase inhibitor[107], results in HIF-1α activation and induces mitochondrial dysfunction, which is reflected by an increase in ROS, mitochondrial membrane potential and mass. In parallel, dimethyloxalylglycine (DMOG) induces a switch to glycolysis and promotes RA FLS invasiveness. Manipulation of prolyl hydroxylases in vascular models has highlighted their critical involvement in the regulation of the maturity, stability and survival of blood vessels[108]. Owing to its avascular nature, cartilage is considered to be an oxygen-deprived tissue. Nevertheless, human chondrocytes are able to enhance matrix synthesis in hypoxic conditions through the HIF-1α-mediated upregulation of SOX9 (REF. 109); HIF-1α stability, in turn, is mediated by the inhibition of PHD2 in response to low pO_2. These data are consistent with the protective effects conferred by prolyl hydroxylase inhibitors in other diseases such as colitis[110].

NF-κB signalling

As one of the key transcriptional pathways in RA, NF-κB signalling primarily regulates the levels of proinflammatory mediators and components of anti-apoptotic pathways. In the canonical pathway, the activity of NF-κB is controlled by inhibitor of NF-κB (IκB) kinase (IKK), which mediates the serine phosphorylation and degradation of IκBα, the endogenous inhibitor of NF-κB; degradation of IκBα induces nuclear translocation of NF-κB.

Previous studies have shown that NF-κB can promote HIF-1α activation in response to proinflammatory cytokines[111,112]. NF-κB binding sites are present within the HIF-1α promoter, the mutation of which results in the loss of HIF-1α activation in response to hypoxia[113].

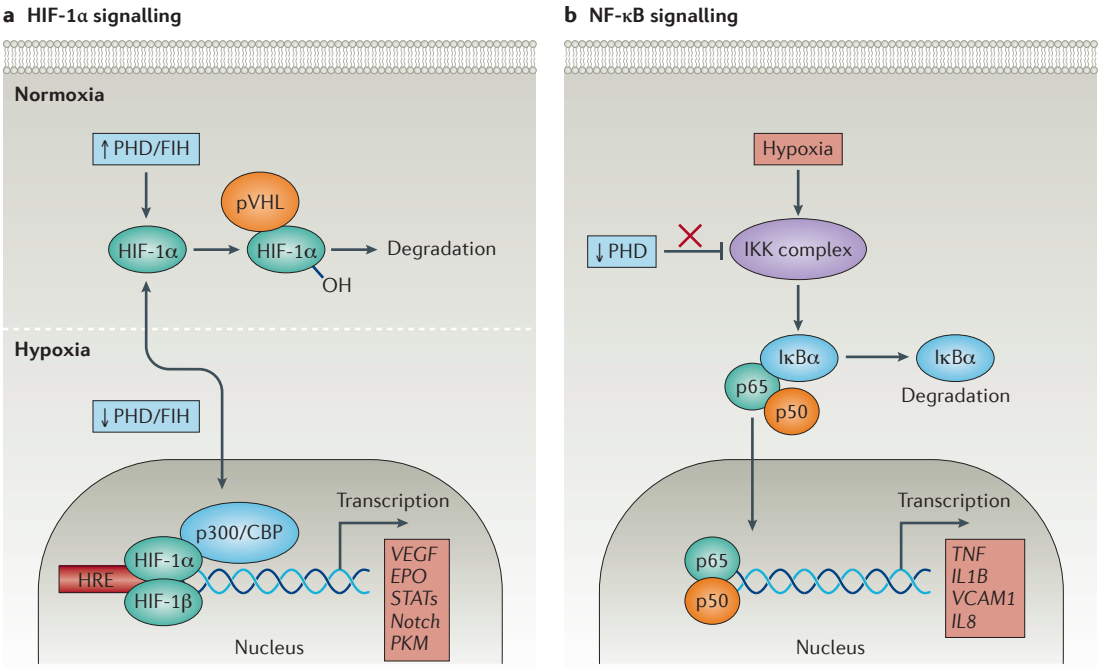

Figure 4 | **Hypoxia-induced signalling in the rheumatoid joint. a** | Under normoxic conditions, hydroxylation of hypoxia-inducible factor (HIF)-1α by prolyl hydroxylases (PHDs) generates a binding site for the von Hippel Lindau tumour suppressor protein (VHL), thereby promoting the ubiquitylation and subsequent proteasomal degradation of HIF-1α. However, under hypoxic conditions, such as in the inflamed joint, the hydroxylation activity of PHDs is reduced, resulting in the accumulation and activation of HIF-1α, which can then translocate into the nucleus and associate with HIF-1β and the cofactor p300/CBP. This complex binds to, and induces the transcription of, genes containing hypoxia-responsive elements (HRE), such as *VEGF*, *EPO*, *STAT3*, *Notch* and *PKM2*. **b** | Nuclear factor κB (NF-κB) is one of the main transcription factors in the inflamed joint. Both inflammatory stimuli, such as cytokines, and hypoxia can activate NF-κB signalling. Under hypoxic conditions, the PHD-mediated repression of inhibitor of κB kinase (IKKβ) is suppressed, which leads to increased IKKβ activity and enhanced phosphorylation of inhibitor of κBα (IκBα); IκBα phosphorylation causes its degradation, enabling NF-κB subunits (p65 and p50) to translocate to the nucleus, where they activate the transcription of inflammatory genes such as *TNF*, *IL1B*, *VCAM* and *IL8*. EPO, erythropoietin; FIH, asparagine hydroxylase factor inhibiting HIF; PKM2, pyruvate kinase M2; STAT, signal transducer and activator of transcription.

Furthermore, activation of the NF-κB p65 subunit is associated with low *in vivo* synovial pO_2 levels in RA synovial biopsy samples[114]. NF-κB–HIF-1α signalling also mediates the synergistic effects of IL-17A and hypoxia on the invasiveness of FLSs[98]. In osteoclasts, the expression of VEGF induced by receptor activator of NF-κB ligand (RANKL) is mediated through NF-κB and subsequent induction of HIF-1α. The increase in VEGF promotes angiogenesis, facilitating leukocyte infiltration and further promoting an hypoxic environment[115]. Furthermore, inhibiting NF-κB in a CIA model inhibits HIF-1α activation, angiogenesis and synovial inflammation[116].

The mechanism(s) by which hypoxia influences NF-κB in the inflamed joint is unclear, but studies in other cell types have shown that, under hypoxic conditions, prolyl hydroxylases show decreased hydroxylation activity towards IKKβ, which thereby relieves the repression of NF-κB[117] (FIG. 4). Consistent with this mechanism, IKK2 contains an evolutionarily conserved LxxLAP consensus motif within its activation loop, which can potentially be hydroxylated by prolyl hydroxylases. Furthermore, the ankyrin-repeat domains of the p105 precursor of the p50 subunit of NF-κB and IκBα are hydroxylated by FIH, indicating that prolyl hydroxylases are directly involved in the NF-κB transcriptional response[118].

Notch signalling

Notch receptors and their ligands are transmembrane proteins that regulate cell fate decisions, proliferation, differentiation and apoptosis. Following cleavage by γ-secretase, the intracellular domain of Notch, NICD, translocates to the nucleus, where it stimulates the transcription of the 'hairy/enhancer-of-split' (*Hes*) and Hes-related transcription factor (*Hrt*) families of transcriptional repressors[119]. Under hypoxic conditions, Notch signalling is a key mechanism involved in the differentiation, in angiogenesis, of vascular-tip versus stalk cells, during which the tip cells guide the vascular sprouts followed by the stalk cell, which forms a functional vascular branch, and then the phalanx cells, which are involved in the developing plexus[120]. The Notch ligands DLL4 and Jagged-1, NICD, and downstream target genes have all been detected in RA synovial tissue[107]. In the inflamed joint, NICD signalling mediates angiogenesis induced

by VEGF or Ang2 as well as regulating chondrocyte proliferation. High levels of NICD are associated with low levels of pO_2 in synovial tissue *in vivo*, and its levels, along with the expression of *DLL4*, *HEY1* (encoding HRT-1), and *HEY2* (encoding HRT-2), are induced in RA FLSs and human dermal microvascular endothelial cells in response to hypoxia[107], which is consistent with studies showing the presence of HIF-1α binding domains and HREs in the promoters of *HEY1*, *HEY2* and *DLL4* (REF. 121). In turn, HIF-1α activation can be inhibited in the presence of the Notch inhibitor DAPT in RA cells[107]. HIF-1α and NICD have been demonstrated to interact, increasing the stability of HIF-1α under hypoxic conditions[122]. However, the HIF-1 inhibitor FIH not only binds HIF-1α, but can also bind Notch, and shows a higher affinity for Notch than it does for HIF, suggesting that Notch might regulate HIF-1α activation by sequestering FIH[123] (FIG. 5).

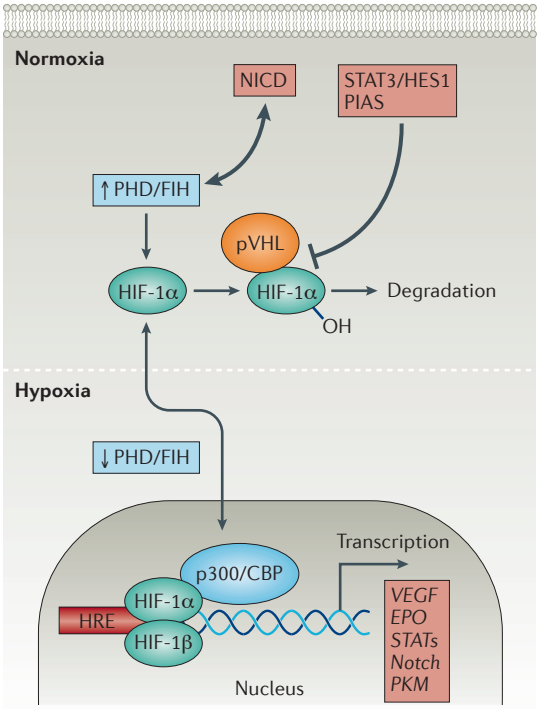

Figure 5 | **Interplay between HIF-1α, Notch and STAT3 signalling.** Interplay exists between hypoxia-inducible factor (HIF)-1α, the Notch intracellular domain (NICD) and signal transducer and activator of transcription (STAT)3 signalling at various levels. Hypoxia-induced STAT3 can, in turn, accelerate the activation of HIF-1α, an effect mediated through competition of STAT3 with HIF-1α for binding to the von Hippel Lindau (pVHL) protein, which might involve the Notch-1 downstream target gene *Hes1*. Protein inhibitor of activated STAT (PIAS), a negative regulator of STAT activation, also has the ability to interact with pVHL to induce VHL SUMOylation and subsequent inactivation, thereby preventing pVHL from promoting the ubiquitylation and proteasomal degradation of HIF-1α. The prolyl hydroxylase FIH can hydroxylate the ankyrin repeat domain of Notch and displays a higher affinity for Notch than for HIF, suggesting that Notch might sequester FIH activity, thereby allowing the accumulation and activation of HIF-1α. FIH, asparagine hydroxylase factor inhibiting HIF.

JAK–STAT signalling

The Janus kinase (JAK) family of receptor-associated tyrosine kinases has been implicated in RA; once activated, JAKs recruit and activate signal transducers and activators of transcription (STATs), which, in turn, drive gene transcription[124]. In RA, STAT activation correlates with synovitis, promotes FLS survival[125] and mediates RANKL-dependent osteoclastogenesis[126], whereas JAK2 inhibition ameliorated disease in CIA and in collagen-antibody-induced arthritis models[127]. *In vivo*, synovial pO_2 levels induced STAT3 activation and promoted migration and invasion in RA FLSs. However, although HIF-1α silencing inhibited these effects, STAT3 blockade could also inhibit hypoxia-induced HIF-1α expression, which suggests the existence of bi-directional interactions between STAT3 and HIF-1α[128]. These results are consistent with studies showing that HIF-1α facilitates the binding of STAT3 to the haptoglobin promoter in human hepatoma cells[129]. Hypoxia-induced STAT3 activation can accelerate the accumulation and activation of HIF-1α[130]; STAT3 binding to HIF-1α inhibits the interaction of HIF-1α with, and subsequent ubiquitylation by, VHL[131] (FIG. 4). PIAS, a negative regulator of STAT activation that is known to be expressed in the inflamed synovium, can also interact with VHL and induce VHL SUMOylation, thereby inactivating it[132]. Further studies have shown that activation of hypoxia signalling in cancer cells is Hes1/STAT3 dependent[133], that the JAK inhibitor WP1066 significantly decreases hypoxia-induced HIF-1α–NICD signalling in RA[131], and that inhibition of STAT3 reduces hypoxia-induced Notch activation in glioblastoma stem cells[134]. Furthermore, Jagged-1–Notch-mediated regulation of inflammatory responses is mediated through both the NF-κB and JAK–STAT–SOCS signalling pathways[135]. Together, these studies demonstrate complex interactions between key signalling pathways within the inflamed hypoxic synovial microenvironment, and provide evidence for both positive and negative feedback loops at many different levels. Increased efficiency of the oxygen supply to the synovium might therefore lead to inhibition of these pathways and promote resolution of inflammation.

Therapeutic implications

Several studies have examined the consequence of blocking angiogenic pathways such as those mediated by VEGF, its receptors or the angiopoietins in models of arthritis, both *in vitro* and *in vivo*, and have reported a delayed onset of arthritis, reduced number of synovial blood vessels and cellular infiltrates, and decreased joint damage[10,11,137,140]. Bevacizumab, an anti-VEGF monoclonal antibody[141], has been approved for the treatment of colon, kidney and lung cancer; however, its efficacy has not been demonstrated in RA. Several pharmacologic agents that activate or inhibit HIF signalling have also been examined in many disease states. Activators of HIF, including hydroxylase inhibitors such as DMOG, FG-4497 and JNJ1935, have been shown to have beneficial effects in *in vitro* and *in vivo* studies on epithelial and endothelial cell function in vascular and colitis models[120,142,143]. In the context of RA, however, molecules that promote, rather than inhibit,

hydroxylase activity — specifically, the activity of PHD2 — are likely to promote HIF-1α-hydroxylation and its subsequent proteasomal degradation[108], resulting in inhibition of synovial angiogenesis and leukocyte infiltration, and thus the prevention of joint damage. Small molecular weight molecules that interfere with HIF-1 processing and activation that might have a beneficial therapeutic effect include temsirolimus and HSP90, which have been shown to inhibit cellular proliferation, growth and survival of tumour cells[144,145].

Tofacitinib, a JAK1 and JAK3 inhibitor approved for the treatment of RA, can inhibit HIF-1α signalling, angiogenic mechanisms and proinflammatory mediators in *in vitro* and *in vivo* models of RA. Studies have shown that pyruvate kinase M2 (PKM2), which is responsible for catalysing one of the last steps in glycolysis, directly activates STAT3, which subsequently activates HIF-1α, thereby creating a PKM2–STAT3–HIF-1 feedback loop[146] (as PKM2 is induced in response to HIF-1α). Small molecule activators of PKM2 such as TEPP46, which promotes PKM2 tetramerization, have been shown to have anti-tumour effects in mice. Tetramerization inhibits translocation of PKM2 into the nucleus and thus inhibits its ability to activate HIF-1α[147]. Rapamycin targets mTOR, which, in response to environmental cues, promotes either cellular growth or catabolic processes during conditions of stress. mTOR has a critical role in directing T-cell differentiation and function, and its inhibition promotes the generation of T_{REG} cells[148]. mTOR is also a major repressor of autophagy, indicating that pharmacological enhancement of autophagy might have disease-modifying activity in the treatment of RA. In mice with experimentally induced osteoarthritis, systemic administration of rapamycin has been shown to induce autophagy, which is associated with a decrease in the severity of synovitis and a reduction in the expression of IL-1β[149]. Furthermore, mTOR deletion protects mice from osteoarthritis by upregulating autophagy and significantly reducing articular cartilage degradation and synovial fibrosis[150]. Finally, metformin, the most widely used anti-diabetic drug, acts in part by indirectly activating the energy sensor AMPK, which can inhibit mTOR and HIF-1α; metformin has been shown to attenuate disease in mouse models of autoimmunity including in collagen-antibody-induced arthritis[151]. In the K/B×N arthritis model, metformin-mediated inhibition of mTOR activity enhanced autophagic flux, resulting in the suppression of NF-κB signalling and inflammatory cytokine production, thereby highlighting modulators of autophagy as potential therapeutic agents for the treatment of RA[152].

Conclusion

The RA joint is profoundly hypoxic as a result of dysregulated angiogenesis, impaired mitochondrial function and inflammation, which leads to a bioenergetic crisis. Synovial cells show adaptive survival responses when exposed to hypoxia, which, in conjunction with altered metabolism, activate and regulate key transcriptional signalling pathways in the RA joint to further exacerbate inflammation. This metabolic change is common in many inflammatory diseases, so understanding the regulation of hypoxia-mediated pathways in inflammation, and the resolution of this process, could provide a basis for novel therapies. The newly discovered characteristics of mitochondria as DAMP-releasing organelles suggests they have a role as central modulators of inflammation; however, the precise mechanism by which damaged mitochondria might activate the immune system needs to be elucidated. It also remains unclear which types of cell in the hypoxic joint are responsible for the release of mitochondrial DAMPs, and whether DAMPs released from different types of cell might have different biological roles. Future research will involve the in-depth characterization of immune cells at the site of inflammation — in particular, DCs, macrophages and T cells, which exhibit a high degree of plasticity. Characterizing cells using surface markers is no longer sufficient for identifying immune populations, especially in the context of inflammatory tissues. Gene-expression profiling techniques such as RNA-Seq will need to be used to characterize the functional properties of cells at the site of inflammation, to determine their origin and to provide insights into their development. With this knowledge, targeting destructive inflammatory cells without affecting the overall immune repertoire might be possible. Owing to the dysregulated metabolic nature of these cells in autoimmunity, the use of glycolytic inhibitors also represents a promising avenue of research. Indeed, it is well appreciated that many of the proinflammatory and proliferative functions of inflammatory-immune cells are associated with high levels of glycolysis, suggesting that reverting the metabolic profile back to oxidative phosphorylation might limit the inflammatory potential of these cells and provide a novel therapeutic approach for RA.

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Author contributions

All authors researched data for article and made substantial contribution to discussions of the content, writing and review/editing of the manuscript before submission.

Competing interests statement

The authors declare no competing interests.