

Review

Novel Targeted Therapies for Rheumatoid Arthritis Based on Intracellular Signalling and Immunometabolic Changes: A Narrative Review

Marveh Rahmati^{1,*}, Maria Paula Kwesiga², Jiachen Lou^{3,4}, Ai Lyn Tan^{4,5},
Michael F McDermott^{4,*}¹Cancer Biology Research Center, Cancer Institute, Tehran University of Medical Sciences, 14166-14178 Tehran, Iran²Department of Biomedical Sciences, Grand Valley State University, Allendale, MI 49401, USA³Faculty of Biology, Medicine and Health, The University of Manchester, M13 9PL Manchester, UK⁴Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, LS9 7TF Leeds, UK⁵NIHR Leeds Biomedical Research Centre, Chapel Allerton Hospital, Leeds Teaching Hospitals NHS Trust, LS7 4SA Leeds, UK*Correspondence: m_rahmati@tums.ac.ir (Marveh Rahmati); M.McDermott@leeds.ac.uk (Michael F McDermott)

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Abstract

Rheumatoid arthritis (RA) is a relatively common systemic autoimmune disease with an estimated prevalence of approximately 1% worldwide. Patients present predominantly with symmetrical small joint inflammatory arthritis, which involves dysregulated immune responses, leading to bone and cartilage deformities due to extensive erosive damage. The introduction of biological based therapies for the management of this life-altering condition, over the past three decades, has led to marked improvements in patients' quality of life. A wide range of both innate and adaptive immune cells are involved in the pathogenesis of RA, with a complex interplay of cytokines, T-cells, B-cells, and dendritic cells. Some of these cells have been successfully targeted in the treatment of RA by the use of biologics-based therapies. For example, rituximab therapy blocks B cell activation and abatacept effectively blocks T cell activation in patients with RA. Despite these advances, there remain some patients who are resistant to all current therapeutic options, which has encouraged further research into understanding the primary signal transduction pathways that mediate the disease. In this review we discuss the roles of the main signalling pathways, including metabolic reprogramming that have been implicated in RA disease progression, in order to develop a conceptual framework for more precise deployment of existing therapies, and to provide a rationale for producing molecular inhibitors of these pathways. Improved knowledge of the many intracellular signalling pathways in RA will complement current precision medicine strategies, particularly for the patients with difficult-to-treat RA, and especially in those with multidrug resistance disease.

Keywords: rheumatoid arthritis; intracellular signalling pathways; ER stress; treatment response; multidrug resistance; autoimmune/autoimmunity; inflammation; refractory RA; metabolic reprogramming/metabolites

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune and inflammatory disease, characterised by recurrent episodes of inflammatory arthritis, which are often symmetrical, involving peripheral joints with the hands, feet and wrists being the most commonly affected. Active disease in RA implicates both innate and adaptive elements of the immune system with persistent inflammation of synovial tissue and extra-articular organ involvement, more frequently seen in patients with severe disease. Regulatory mechanisms normally used by the body to contain self-directed immune responses may be insufficient to regulate the production of self-reactive autoantibodies like rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA) also known as anti-cyclic citrullinated peptide antibodies (anti-CCP) [1,2]. This inappropriate activation of B cells and dysregulated immune responses may eventually result in marked deformities of bone and cartilage, due to extensive erosive damage, mainly in the smaller joints of

the hands and feet, with associated systematic symptoms, such as fatigue, general malaise and musculoskeletal pain. The opportunity to target these pathways has provided patients with therapeutic hope. However, despite the increasing knowledge in treating patients with RA, disappointingly, there are still patients who do not have favourable responses to existing therapies. In this review, we explore the pathogenesis of RA, and discuss the roles of the main signalling pathways, including metabolic reprogramming, that have been implicated in RA disease progression in order to develop a conceptual framework for more precise deployment of existing therapies, and also to provide a rationale for producing molecular inhibitors of these pathways. Improved knowledge of the many intracellular signalling pathways in RA will complement current precision medicine strategies, particularly for patients with difficult-to-treat RA, and especially for those with multidrug resistance disease.



2. Methodology

Different databases, including Pubmed, Web of Science, SCOPUS and Google Scholar, were used for the keywords of 'Rheumatoid arthritis, Intracellular signalling pathways, endoplasmic reticulum (ER) stress, Treatment response, Multidrug resistance, Autoimmune/autoimmunity, Inflammation, Refractory RA, Metabolic reprogramming/metabolites'. Inclusion criteria included all of the following keywords; RA pathogenesis, inflammation, immune tolerance, janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, stress-activated protein kinases/mitogen-activated protein kinases (SAPK/MAPK) pathway, PI-3K/AKT/mTOR pathway, Interferon signalling pathway, ER stress and the unfolded protein responses (UPR) pathway, vascular endothelial growth factor (VEGF) signalling pathway, spleen tyrosine kinase (SYK) signalling pathway, Wingless/Integrated (Wnt) signalling pathway, Notch signalling pathway, metabolites and pathogenesis, response to treatment and drug resistance. Exclusion criteria were the studies that included other rheumatological diseases apart from RA.

3. Rheumatoid Arthritis and Its Pathogenesis

RA is a heterogenous condition and ACPA/anti-CCP autoantibodies, which are a hallmark of RA, are present in approximately 70% of these patients, in most reported series [3]; the specificity of ACPA/anti-CCP antibodies for RA ranges between 87% to 98% which makes their presence an excellent diagnostic indicator/marker of this disease. Shared epitope positive HLA-DR alleles are associated with anti-citrullinated peptides and with genetic susceptibility to developing RA. However, the molecular basis for these two associations is unknown.

The process of citrullination refers to post-translational conversion of arginine to citrulline residues by peptidylarginine deiminase enzymes (PADs) and is a key factor in the pathogenesis of RA [4]. The mechanisms controlling the citrullination process in RA are both dysregulated and upregulated. Under normal circumstances, the citrullinating enzymes are controlled by levels of calcium and redox conditions in order to limit their hyperactivation. Perforins and bacterial toxins induce prominent calcium influx which induces the hyperactivation of PADs and hypercitrullination in the RA joint. In addition, the extracellular environment of synovial joints in RA is in a state of oxidative activation [5], which is known to inactivate PADs: efficient extracellular citrullination in RA is likely to require the constant release of active enzymes from dying cells, which may be accelerated in the presence of PAD-activating autoantibodies.

PADs are inactivated extracellularly, and the presence of PAD activating co-factors, such as anti-PAD activating antibodies, and the continuous release of active PADs enzymes from dying and activated cells are necessary for effi-

cient extracellular citrullination in RA. Therefore, targeting the signalling pathways that lead to PAD enzyme hyperactivation has the potential to prevent the development and progression of the RA citrullinome, with consequent clinical improvement in patients with RA [6].

An early study has suggested that one citrullinated peptide from vimentin, Vim R70Cit, could bind shared epitope positive with greater affinity than shared epitope negative HLA-DR molecules thus presenting citrullinated peptides to T cells that help production of IgG ACPA [4]. However, a thorough investigation of the binding of 180 peptides, either citrullinated or native, encompassing the entire alpha and beta chains of human fibrinogen did not find that citrullinated peptides bound shared epitope positive HLA-DR molecules better than their native counterparts. Besides, half of the patients with RA have IgG autoantibodies to the peptidyl arginyl deiminase 4 (PAD4) enzyme. This suggests the presence, in these patients, of helper T cells recognizing peptides from PAD4, which could provide help to B cells specific for citrullinated epitopes present on proteins being citrullinated by PAD4, by a hapten-carrier mechanism [7].

The main cause(s) of RA is unknown; however, some genetic and environmental factors are definitely involved in initiating disease onset [8]. Studies on patients with RA have revealed genetic factors that underlie the disease, such as the human leukocyte antigen (HLA)-DRB1 locus, where some alleles contain a five amino acid motif sequence (QKRAA), in residues 70–74 in the HLA-DRB1 region, named the shared epitope. Other factors include selection of certain predisposing T-cell repertoires, and antigen presentation; alteration in peptide affinity may also lead to autoreactive immune responses in RA [9]. The prognosis in patients with RA who are seropositive for ACPA and/or RF, is worse than those with ACPA-negative disease [10]. Neuro-immunological factors also influence disease development [8].

The environmental risk factors which are reported in patients with RA include smoking, exposure to silica, periodontitis, and gut microbiota. Another risk factor in RA is gender, as the disease is three times more common in women than in men [9].

The genetic and environmental risk factors as well as epigenetic elements lead to post-transcriptional modifications and alteration in self-protein citrullination. Anti-citrullinated peptides such as α -enolase, keratin, fibrinogen, fibronectin, collagen, and vimentin are recognised as foreign antigen in the sera of patients [11]. Infectious agents and the gut microbiome are also associated with progression of the disease [12]. In patients with RA, dysregulation of the gut microbiota can affect the host immune responses which leads to disease development. The gut microbiota also can be used for prediction of the susceptibility of patients to RA and may be considered as have potential targets to control the incidence of RA. The enzymes and prod-

ucts of gut microbiota also affect the toxicity or efficacy of drugs, either directly or indirectly. On the other hand, different medications can modulate the gut microbiota composition; the administration of etanercept, which contains the TNF receptor, may restore the beneficial microbiota [13].

Variations in specific microbiota are proven to be associated with both disease development and response to drugs. In this regard, specifically targeting the gut microbiota in each individual patient may alleviate RA disease activity, increase drug efficacy and improve the outcome [14]. The different gut microbiota composition in patients with RA, compared to healthy controls, shows the importance of gut dysbiosis in the onset of RA. One of the most important environmental factors that modulate the gut composition is nutritional content of the diet [15].

To prevent and manage the degree of inflammation in RA, modulation of the gut microbiota through diet is recommended. For instance, the Mediterranean diet has beneficial effects on the gut microbiota and may improve the outcome in RA [16]. The use of *Lactobacillus acidophilus* and *Lactobacillus casei* in probiotic products are also helpful in reducing disease activity while bacillus spp. and other lactobacillus spp. are increased in quantity in the gut of patients with RA. It seems that different lactobacilli have different roles in RA pathogenicity and diseases activity [14]. In addition, faecal microbiota transplantation (FMT) from healthy individuals to patients with RA may also reduce disease activity [17]. Furthermore, the content of gut microbiota could potentially be considered as biomarkers for RA diagnosis, especially in early onset disease. The abundance of beneficial intestinal bacteria, such as Bacteroidetes, is decreased in early RA while *Prevotella copri* are increased and take the place of other beneficial bacteria [18]. Not only gut microbiota but also the oral microbiota are important in RA development. Dysbiosis of oral microbiota may lead to periodontitis and the inflamed gingiva contains citrullinated proteins and ACPA. *Porphyromonas gingivalis*, a common microorganism in gingiva, can citrullinate the proteins for ACPA development, and is involved in the onset of RA disease [19].

A normal synovium contains mesenchymal-derived fibroblast-like synovial cells (FLS) and macrophages. In RA the FLS acquire specific characteristics, including increased proliferation, apoptosis resistance, as well as secretion of high-level disease-associated cytokines and chemokines, adhesion molecules, matrix metalloproteinases (MMPs), and tissue inhibitors. FLS are directly involved in cartilage destruction and chronic inflammation [20].

Considerable insights into the pathogenesis of RA may be gained from study of the mechanisms of action of drugs shown to be effective in RA. Methotrexate (MTX) is a conventional synthetic disease modifying antirheumatic drug (csDMARD) that is often used as a first line treatment for RA. MTX is a folic acid antagonist that inhibits the syn-

thesis of both DNA and RNA and exerts anti-inflammatory effects on many pathways involved in RA. Furthermore, MTX works synergistically with different anti-TNF therapies, independently of its property of reducing the titres of anti-drug antibodies [21].

4. Inflammation and RA

The balance between pro- and anti-inflammatory cytokine levels is disturbed in rheumatoid joints, leading to the process of autoimmunity, chronic inflammation, and associated joint damage. Inflammation of the synovium begins with infiltration, accumulation, and migration of leukocytes into synovial micro-vessels, which induce the expression of adhesion molecules such as integrins, selectins, in addition to members of the immunoglobulin superfamily and chemokines. These adhesion molecules and chemokines facilitate leukocyte rolling, adhesion, and transmigration into the synovial tissue. Within the synovium, leukocytes release proinflammatory cytokines, such as TNF, IL-1 β , and IL-6, which further activate synoviocytes to perpetuate the inflammatory response with production of more cytokines, chemokines, and matrix-degrading enzymes, all leading to joint destruction and cartilage damage. This persistent inflammation and joint damage may ultimately result in chronic joint disease as found in RA [22]. Self-directed immune responses induce autoantibodies to trigger an adaptive response with increased level of T-cells against citrullinated self-proteins [23]. T-cell differentiation towards a TH1/TH17 phenotype is linked to the presence of IL-15, IL-1, IL-6, TGF β , IL-12, and IL-23 in synovial tissue [24]. B-cells in the synovium also contribute to antigen presentation and cytokine secretion [25]. Both macrophage-derived and dendritic-cell-derived transforming growth factor β as well as interleukin-1 β , 6, 21, and 23 trigger Th17 differentiation and prevent the production of regulatory T cells (Treg) [26]. This imbalance between Th17 and Treg induces TNF, which further inhibits Treg activity [27]. Furthermore, the macrophage-derived cytokines including TNF, IL-1, IL-6, IL-15, and IL-18 also stimulate many proinflammatory cytokines in synovial tissue, where myeloid cells, plasmacytoid dendritic cells (pDCs) release cytokines such as IL-12, 15, 18, and 23 and HLA class II molecules, as well as costimulatory molecules which may cause further T-cell activation [23,28].

Cytokine production is essential to the development of RA. The early stages of disease show cytokine expression patterns of IL-4, 13, and 15 TNF and IL-6 and during inflammation the other cytokines, chemokines and adhesion molecules are also activated. DCs also have an important role in the initiation and perpetuation of RA through presentation the arthritogenic antigens to T-cells. Two major DC subsets, myeloid DCs (mDCs) and pDCs, in RA synovial tissue are reported to have different cytokine profiles [29]. In RA synovial tissues, both pDCs and mDCs release IL-15 and IL-18, while pDCs express IFN α/β and mDCs

secretes IL-12p70 and IL-23p19 [30]. Moreover, the rate of angiogenesis is increased [31], and IL-6 exerts systemic effects, such as the acute phase response, anaemia, cognitive impairment, and dysregulation of lipid metabolism. IL-1 family cytokines (including IL-1 α , 1 β , 18, 33) promote the activation of leukocytes, endothelial cells, chondrocytes, and osteoclasts in RA [31]. Cytokines are also involved in osteoclast maturation and activation and nuclear factor- κ B ligand-receptor activator (RANKL) plays a central role in this process, along with TNF, IL-17, and IL-1 [22].

5. RA and Immune Tolerance

When loss of immune tolerance to self-antigens occurs in diseases such as RA, protective immune mechanisms switch to auto-aggressive immunity in dedifferentiated synovocytes, with T cell-mediated joint damage and loss of tissue-protective macrophage populations. The Treg subpopulation of helper T-cells prevents the development of autoimmunity and maintains immune tolerance by using a variety of mechanisms, including elimination of self-reactive T cells and production of anti-inflammatory cytokines, such as IL-10 and TGF- β [32].

In effect, Treg cells are divided into three categories according to their origin and differentiation: they are produced by immature T lymphocytes during thymus development, and with a phenotype is CD4⁺CD25⁺Foxp3⁺ T cells, which are called natural Treg (nTreg) cells that constitutively express CD25 and express the specific nuclear transcription factor, Foxp3. Upon peripheral antigen stimulation or immunosuppressive factor induction, mature CD4⁺CD25⁻ T cells are transformed into acquired Treg (iTreg) cells, including Tr1 and Th3 subsets; the former mainly secretes IL-10 and TGF- β , while the latter mostly produces TGF- β . In addition to regulatory CD4⁺ T cells, regulatory CD8⁺ T cells also exist in the CD8⁺ T cell lineage.

In the thymus, nTreg cells are derived from hematopoietic progenitor cells and migrate into the peripheral blood to maintain immune tolerance and to prevent autoimmunity [33].

During the development of peripheral Treg cells, naïve CD4⁺ T cells first migrate into the peripheral blood without any TCR activation. Once stimulated by antigens in the peripheral blood, naïve CD4⁺ T cells can differentiate into Foxp3⁺ Treg cells in the presence of both TGF-beta (TGF- β) and IL-2. Despite their low frequency, peripheral Treg cells can also prevent inflammation developing in barrier tissues. When Treg cells are generated *in vitro*, they are termed iTreg cells. These cells are also suppressive in nature and help maintain immune homeostasis; it should be emphasised in this context that iTreg cells are less stable than nTreg cells.

There is an additional CD4⁺ T cell subset that mediates immunosuppression *in vitro* and which is characterised by secretion of TGF- β 1 and IL-10, without express-

ing the Foxp3 transcription factor. This subset is termed type 1 regulatory T cells (Tr1 cells). However, no surface biomarkers or transcription factors have so far been identified for this specific subset, although some promising candidates have been reported. The suppressive mechanisms, used by Tr1 cells, are similar to those employed by nTreg cells and include the production of immunosuppressive cytokines, cell-cell contact-mediated suppression, cytotoxicity, and metabolic disruption. IL-10 and other cytokines, including IFN- α , IL-6 and IL-27, are required for the generation of Tr1 cells. Importantly, Tr1 Treg cells are reported to be less suppressive than nTreg cells in the early stage of life as they fail to rescue IPEX patients, who have a complete lack of Foxp3. In addition, it has been reported that Foxp3⁺ CD8⁺ T cells, CD4⁻CD8⁻ cells and gamma/delta T cells all share some suppressive properties, but no evidence has produced to show that they play a significant role in self-tolerance [34].

The mechanisms of self-tolerance are complicated, however, and dysfunction of Treg cells and dendritic cells (DCs) are among the proposed mechanisms underlying the breakdown of self-tolerance in the pathogenesis of RA. Furthermore, the ratio of Treg/Th17 and Th1/Th2 cells is vitally important and the imbalance between these specialised subpopulations of T cells induces the initiation of RA pathology [35]. The anti-TNF biologic, etanercept, in combination with MTX has been shown to ameliorate disease in RA by normalising the distribution of Th17 and Treg [36] cells. In a number of chronic autoimmune diseases, enhancement of Treg function by the use of tolerogenic dendritic cells or stem cell transplantation are both potentially effective treatments to re-establish immune tolerance [37,38]. Moreover, tolerogenic vaccine platforms to deliver the self-antigens to specific antigen-presenting cell subtypes are other therapeutic strategies that may be employed for treat RA [22].

In autoimmunity, it has been postulated that citrullination of peptides and self-antigens may expose cryptic peptides by modifying protein structure and proteolytic cleavage, with subsequent changes to antigen processing by the MHC-II, which may, in turn, lead to loss of immune tolerance to self-antigens because of exposure of these cryptic peptides previously hidden from immune system [6].

6. Intracellular Signalling in RA Pathogenesis

6.1 The JAK/STAT Pathway

Janus kinases (JAKs) belong to the family of tyrosine kinases (TYKs); the basic structure of all JAKs consists of four structural domains consisting of seven homologous regions [JH1-7] [39]. The signal transducer and activator of transcription (STATs) are recruited by their corresponding JAKs to act as transcription factors to orchestrate their diverse biological functions (Fig. 1). In summary, the whole signalling module provides a rapid membrane-to-nucleus transmission of the cytokines' signal from the cellular re-

ceptor (e.g., IL-2R) and JAK/STAT signalling is a key intracellular cascade in response to cytokines and growth hormones [40]. This pathway is initiated upon binding the type I/II cytokine to its receptor. Type I/II cytokine receptors are composed of different chains that oligomerize after binding the cytokines [41]. The oligomerization of the four chains separates the intracellular subunits of cytokine receptors, thereby cleaving receptor-associated JAKs from each other and reversing their constitutive inhibition and activation [39]. Autophosphorylation of JAKs converts the inactive form of cytosolic STAT monomers to their active forms, as either homodimers, heterodimers, or tetramers. Active STATs then translocate to the cell nucleus where they function as transcription factors and regulate gene expression [41]. In RA, proinflammatory cytokines, including IL-2, IL-3, and IL-19/IL-20, are the main effectors of the JAK/STAT pathway. However, IL-17A, IL-19, and IL-20 also play a significant role in the context of the JAK/STAT pathway activation. IL-17A has been shown to increase matrix metalloproteinases (MMPs)-1, -2, -3, -9, and -13 which are important mediators of the degradation of articular cartilage extracellular matrix (ECM) proteins in RA [42]. MMP-3 plays a central role in this regard as it promotes structural damage in the joints of patients with RA due to its ability to cleave ECM proteins [42]. The expression of the enzyme matrix metalloproteinase-3 (MMP-3) is observed in FLS which is involved in joint destruction in patients with RA. The MMP-3 enzyme degrades collagen types II, III, IV, IX and X, proteoglycans, fibronectin, laminin, and elastin. In addition, MMP-3 can also activate other MMPs such as MMP-1, MMP-7, and MMP-9, rendering MMP-3 crucial in connective tissue remodelling. MMP-3 is abundant in the serum of patients with RA and considered a biomarker of the condition [43,44].

As with the use of the biologic-based (bDMARDs) drugs, the targeted synthetic (tsDMARDs) therapies have also transformed the management of RA. The orally bioavailable, synthetic small-molecule Janus kinase (JAK) inhibitors (JAKi) are examples of tsDMARDs used to treat RA. Clinical trials have shown that tofacitinib, a JAK inhibitor, used either as a monotherapy or in combination with csDMARDs, is effective in the treatment of patients with RA [45]. However, an increased incidence of major adverse cardiovascular events (MACEs) and malignancy has been reported with tofacitinib compared to TNF inhibitors [46]. A dose-dependent increase in venous thromboembolism (VTE) risk was also observed with tofacitinib; these findings have resulted in the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) restricting the use of all JAKi to patients with RA who had failed TNF inhibitors ((i) Janus Kinase inhibitors (JAKi) European Medicines Agency (europa.eu) and (ii) Janus Kinase (JAK) inhibitors: Drug Safety Communication - FDA Requires Warnings about Increased Risk of Serious Heart-related Events, Cancer, Blood Clots, and Death j FDA).

6.2 The SAPK/MAPK Pathway

Mitogen-activated protein kinases (MAPKs) are important signal transduction pathways involved in several intracellular mechanisms such as differentiation, proliferation, and migration in response to extracellular stimuli. MAPK signalling pathways have a dual role in survival and apoptosis which depend on the cell type and the stimulus (Fig. 2). The MAPK family contains at least four subfamilies including, extracellular signalling kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38a/b/g/d) and ERK5. MAPKs are regulated by a three-step cascade consisting of MAPKKK (MAP3K, MEK kinase), MAPKK (MAP2K, MKK, MEK), and MAPK1 [47]. JNKs and p38 signalling pathways contribute to both proapoptotic and anti-apoptotic mechanisms and can also regulate the autophagy pathway [47]. Autophagy has a dual role in cell survival and death during nutrient starvation [48]. In addition, JNK activation decreases ER stress activity and cell death by inducing the expression of anti-apoptotic proteins [49]. The ERK1/2 cascade is a signalling pathway that regulates a large number of cellular processes, mainly by anti-apoptotic functions, including cell proliferation and differentiation [50]. However, there are also several examples of ERK1/2 signalling exerting a role in pro-apoptotic function [51,52].

Stress-activated protein kinases/mitogen-activated protein kinases (SAPK/MAPK) signalling pathways are activated by pro-inflammatory cytokines in RA [42,53] and are considered to be key proteins for targeted therapies [54]. In injured joint tissues, MAPKs are implicated in the regulation of proinflammatory cytokines in response to IL-1, IL-17, and TNF receptors [55]. ERK1/2 also regulates IL-6, IL-12, IL-23 and TNF production in response to lipopolysaccharide (LPS)-stimulated macrophages [56]. Epidermal growth factor (EGF), which is released from FLS, induces ERK1/2 to control the production of cyclooxygenase-2-dependent prostaglandin E2 [57]. A major role of JNK in RA development is the control it exerts on MMP levels and the extent of cartilage destruction. IL-1 β and TNF promote extracellular matrix degradation by activating the JNK downstream signalling pathway and modulating MMP expression in chondrocytes and FLS [58]. The use of p38 inhibitors can block the activity of some cytokines including, TNF, IL-1 β , MMP-1, MMP-3, IL-6, and IL-8. p38 inhibitors are potential therapeutic agents in RA [59], but, so far, treatment with these agents have demonstrated limited efficacy in RA [60].

6.3 PI-3K/AKT/mTOR Pathway

PI3K/Akt/mTOR is an important intracellular signalling network in mammalian cells involved in different cellular functions such as cell proliferation, survival, angiogenesis and differentiation [61]. The pathway is also activated to regulate autophagy during fasting or starvation. Elevated levels of PI3K/Akt/mTOR are reported in several

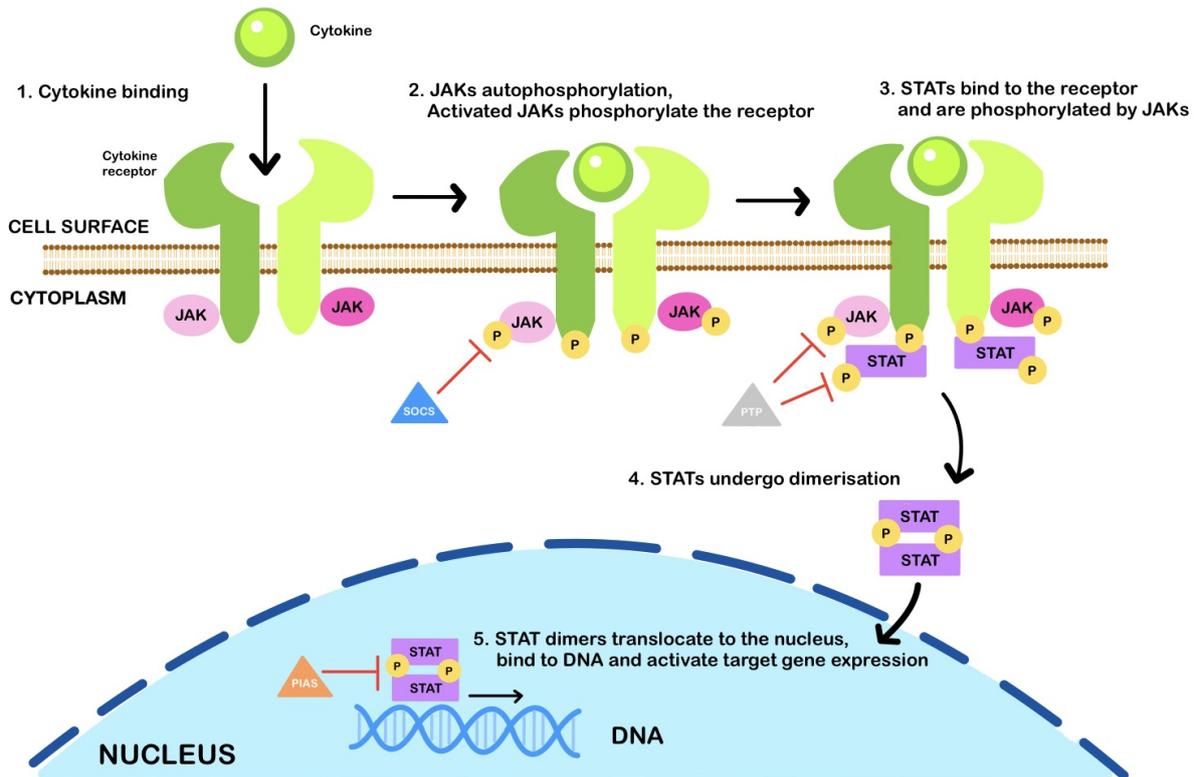


Fig. 1. The JAK-STAT signalling pathway. Janus kinases (JAKs) are activated upon cytokine binding and phosphorylate signal transducer and activator of transcription (STAT) proteins, which dimerise and translocate to the nucleus to regulate gene expression. Inhibitors of the JAK-STAT signalling pathway include suppressor of cytokine signalling (SOCS) proteins, protein tyrosine phosphatases (PTPs), and protein inhibitor of activated STAT (PIAS). [P] is for phosphate.

types of cancers and autoimmune diseases, due to specific mutations and essential proteins such as phosphatase, tensin homologue deleted on chromosome 10 (PTEN), and mTOR [62,63]. During the initiation phase of RA, NGF, PDGF, VEGF, FGF, and cytokines induce PI3K/Akt/mTOR signalling [64,65]. As a result, increased levels of neutrophils, macrophages, eosinophil chemotaxis, mast cell degranulation, and both T- and B-cell infiltration, allied to the proliferation of RA-FLS and increased IL-17 production by CD4⁺ T-cells may occur. The PI-3K/AKT/mTOR pathway may also inhibit immune-cell proliferation by reducing the activity of forkhead box protein O (FOXO) transcription factor [42], which prevent Fas ligand-induced neutrophil apoptosis in inflammatory arthritis [66].

6.4 The Interferon Signalling Pathway

Type I interferons are activated as the first line of the immune response against viruses and microbial products, such as LPS [67]. Theoretically, regulation of the expression of type I interferon might be of therapeutic value, as has been demonstrated for RA [68]. For example, most immune cells, and particularly plasmacytoid dendritic cells (pDCs) release IFN α , whereas fibroblasts, epithelial cells, dendritic cells, phagocytes, and synoviocytes produce IFN β

[69]. Type I interferons bind to a cell surface receptor (IFNAR), composed of the IFNAR1 and IFNAR2 subunits to induce the downstream signalling pathway. This binding causes internalisation of the IFNAR complex which initiates signalling pathways [70] that result in transcription of IFN-stimulated genes (ISGs) [71]. IFNAR activates JAK1 and TYK2 kinases, leading to phosphorylation, dimerisation and nuclear translocation of STAT proteins to regulate gene expression [71]. IFN α activates antigen-presenting cells, boosting CD86 and MHC expression and triggering co-stimulatory signals, which strengthens antigen presentation, links the innate and adaptive immune systems and sets the thresholds against development of autoimmunity [72]. IFN β has both anti-inflammatory and anti-proliferative properties and shares signalling properties with IFN α . Signalling pathways triggered by interferon receptor engagement (e.g., MAPK, NF- κ B, AKT) affect both transcription and translation of ISGs downstream of JAK/STAT pathways [72]. IFN β and TNF together create a delayed antiviral response through a TYK2, STAT2, and IRF9-dependent pathway, independent of STAT1. The type I interferon signature, detected in RA patients' blood, is a biomarker of the preclinical phase of RA [71,73].

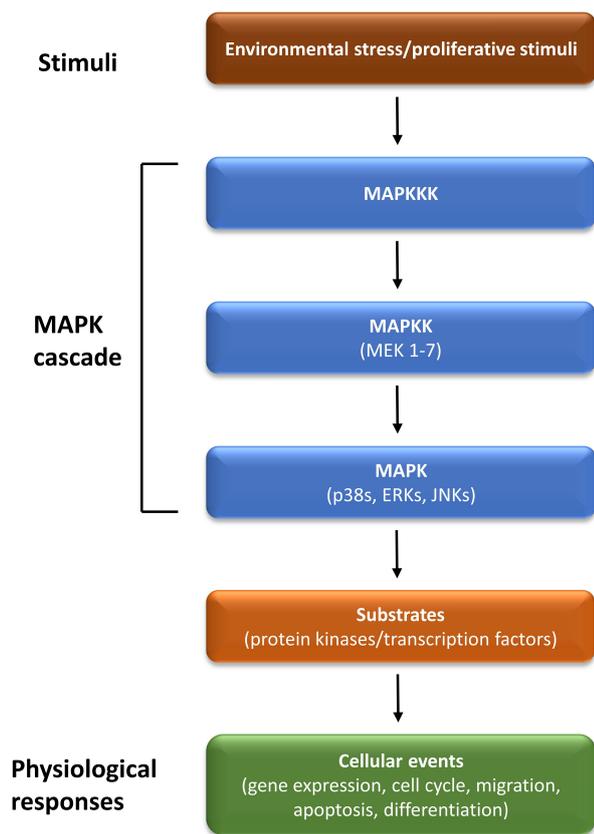


Fig. 2. The MAPK signalling pathway. The mitogen-activated protein kinase (MAPK) cascade consists of three sequentially acting kinases: a MAPK, a MAPK kinase (MAP2K), and a MAPKK kinase (MAP3K). It is activated by a variety of stimuli, and it phosphorylates various substrates, to regulate diverse cellular events. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

6.5 ER Stress and the UPR Pathway

The endoplasmic reticulum (ER), as the largest intracellular membrane-bound organelle in eukaryotic cells, has essential responsibilities in many important cellular functions and interactions, including biosynthesis of lipids, carbohydrate metabolism, Ca^{2+} storage, protein synthesis, and protein modifications. Eukaryotic cells have two different types of ER classified according to the presence of ribosomes; rough ER (RER) has ribosomes attached to the outer surface of the ER, whereas the smooth ER (SER) does not. RER, abbreviated to ER, are involved in the synthesis of membrane or secretory proteins [74]. Newly synthesised proteins, in the ER lumen, are required to fold properly and undergo post-translational modifications, such as the addition of glycan chains or disulfide bridges [75,76].

All proteins that fail to fold properly are retained and removed from the system as they're targeted for degradation by the ER-associated degradation (ERAD) pathway,

by a process of ubiquitylation. Chaperones that reside in the ER assist in folding of the polypeptides. For instance, Hsp70-type chaperone/BiP (binding-immunoglobulin protein, GRP-78) interacts with intact polypeptide chains to prevent their aggregation and facilitates the exquisite protein folding into the correct conformation for their functional structure [77].

Any problems that may arise during protein folding, such as an imbalance between protein synthesis and protein demand, accumulation of misfolded proteins, lack of nutrients, failure of post-translational modification, irregularities of calcium storage and oxygen levels, defects in ERAD and the autophagy pathway may all lead to ER stress (ERS) [78]. The chief mechanism employed by the cells to cope with these various stresses (stressors) is known as the unfolded protein responses (UPR). During an UPR there is upregulation of those proteins which are necessary for protein folding or inhibition of protein synthesis [79].

The UPR is initiated by three ER transmembrane proteins, each having its own signalling pathway, but also with an inherent capacity for crosstalk between these 3 proteins. These three sensors, individually known as inositol requiring enzyme-1 (IRE1, both α and β isoforms), activating transcription factor 6 (ATF6) (both α and β isoforms) and protein kinase RNA-like ER kinase (PERK) [80], each have different potentials to act at different times during their activation. These sensors all bind to BiP in an unstressed situation and under ER stress, these sensors are activated by their dissociation from BiP and each utilises its own signalling pathway to regulate intracellular homeostasis (Fig. 3A).

The PERK sensor is the first to be activated during UPR with dimerisation, oligomerisation, and autophosphorylation. Upon activation, the PERK protein phosphorylates the eukaryotic initiation factor 2 subunit- α (eIF2 α) leading to its inactivation, with inhibition the global translation and repression of protein synthesis [74]. Phosphorylated eIF2 α can also translate some special mRNA species with one or more uORF in their 5'UTR, such as ATF4. ATF4 operates as a transcription factor and modulates various genes involved in different mechanisms including redox homeostasis, amino acid metabolism, protein biosynthesis, programmed cell death (PCD), and autophagy [81,82]. ATF4 is also responsible for inducing the transcription of the mRNA of its targets, growth arrest and DNA damage [GADD]-inducible34 (GADD34), and C/EBP homologous protein (CHOP). GADD34 dephosphorylates the eIF2 α to recover the translation of proteins through its regulatory subunit, protein phosphatase 1 (PP1) [83]. CHOP is a transcription factor involved in regulation of genes that are involved in the apoptosis pathway [84].

IRE1, as a transmembrane protein in the ER, has two domains: the N-terminal, ER luminal domain, senses unfolded proteins, and the C-terminal cytoplasmic region initiates the UPR via serine/threonine kinase and endoribonuclease (RNase) domains. IRE1 then dissociates from the

GRP78 and binds to mal-folded proteins to transmit the signal to the nucleus (Fig. 3A). IRE1 has two isoforms in mammals; IRE1 α and IRE1 β . IRE1 α is mostly involved in the UPR pathway and expressed in every cell, whereas IRE1 β has inherent anti-aggregation activity and less interaction with GRP78. Unlike IRE1 α , IRE1 β directly binds to unfolded proteins rather than interact with GRP78, suggesting a degree of diversity in the sensing of the aggregated proteins sensors [85]. Under ER stress, IRE1 α disassociates from GRP78, oligomerises, and autophosphorylates its own kinase domain. Then, the RNase activity of its domain can remove a 26-nucleotide from the intron of the X-box-binding protein 1 (XBP1) mRNA, thereby shifting the translational open reading frame [86]. The active XBP1 transcription factor, named 'XBP1s' for the spliced format, transfers to the nucleus to upregulate the genes involved in the translocation of ER proteins, folding or secretion of the proteins, as well as elimination of aggregated or misfolded proteins [74]. IRE1 has yet another responsibility in the degradation of some special mRNAs or precursors of microRNAs (miRNAs) within the ER in a mechanism entitled regulated IRE1-dependent decay (RIDD) [87,88]. The purpose of this is to reduce the abundance of mRNAs and excess protein loading [89].

ATF6 acts as both monomer and oligomer to form intra- and inter-disulfide bridges within the ER luminal. Under ER stress, the full format of ATF6 (ATF6p90) moves to the Golgi and is cleaved by two kinds of proteases called site-1 protease (S1P) and site-2 protease (S2P), then is converted to a basic leucine zipper (bZIP) translation figure, named 'ATF6p50'. The spliced ATF6 form can translocate to the nucleus to induce the expression of genes such as ER chaperones and ER protein translocation, as well as proteins involved in folding, maturation and secretion, in addition to proteins involved in the degradation of misfolded proteins [90]. ATF6p50, like XBP1s, enhances the expression of secretory proteins from the ER and Golgi under ER stress [91] (Fig. 3A).

Although the most significant function of IRE1 α is its cytoprotective role, it can also stimulate the apoptotic-signalling kinase-1 (ASK1), which causes activation of downstream of stress kinases, JNK and p38 MAPK that promote apoptosis in situations of severe stresses [92]. IRE1 α signalling is also involved in the development of several immune cell types, including secretory plasma and dendritic cells. IRE1 α -mediated regulation of macrophage polarisation under conditions of metabolic stress has also been reported [93]. In addition to immune cell development, IRE1 α -XBP1 signalling contributes to innate immune responses triggered by various toll-like receptor (TLR) ligands, including lipopolysaccharide (LPS) [94]. Furthermore, IRE1 α activity has also been shown to be upregulated in inflammatory arthritis as well as in lipid-induced inflammation [95]. PERK signalling has both protective and proapoptotic functions in the face of ER stress in

different situation. In chronic ER stress, attenuation of IRE1 signalling is coupled with persistent PERK activity leading to cell death. However, both PERK and IRE1 are activated during acute ER stress. In fact, the switch in IRE1 signalling to PERK activity changes the unfolded protein response (UPR) from being protective to pro-apoptotic in nature. However, in some diseases such as autosomal dominant retinitis pigmentosa, PERK acts as an antagonist of IRE1 leading to disease progression [96]. As loss of PERK leads to IRE1 upregulation and ER-phagy activation, this ultimately results in degradation of the wild type forms of critical proteins, and disease progression [97].

In RA the presence of acute or chronic inflammation, the secretion of excessive amounts of inflammatory cytokines, matrix metalloproteinases (MMPs), and other components of the immune response as well as hypoxia and low glucose levels combine to increase levels of ER stress. To restore the homeostasis, both the ER-associated degradation (ERAD) system and the UPR are activated for maintenance and survival of the cells. In the ERAD pathway, accumulated misfolded/unfolded proteins are transferred to the cytosol and ubiquitinated through E2 (ubiquitin-conjugating enzyme) and synoviolin (E3 ubiquitin ligase) in order to be degraded by the cytosolic 26S proteasome system. In the UPR pathway, the three separate branches, IRE1 α , PERK, and ATF6, combine to inhibit protein translation, increase the expression of various ER chaperones, and maintain pro-survival factors, in order to resolve the ER stress. Of the three arms of the UPR, IRE1 α is the most important and is involved in inflammatory responses [98]. Under ER stress, BiP is dissociated from IRE1 α and attached to misfolded proteins. Oligomerisation and autophosphorylation of IRE1 α then leads to activation of the RNase domain to splice the mRNA of XBP1. The spliced form of XBP1 (XBP1s) acts as transcription factor to stimulate the genes involved in TLR-mediated proinflammatory cytokine release, such as IL-6 and TNF, which may accentuate the level of inflammation in patients with RA. On the other hand, IRE1 α can also interact with TRAF2 and activate I κ B kinase (IKK). Activated IKK leads to the dissociation of I κ B from NF- κ B, and NF- κ B then translocates to the nucleus to induces the genes that regulate production of proinflammatory cytokines, chemokines, and matrix-degrading enzymes. The IRE1 α -TRAF2 complex also activates c-JNK, and consequently activates activator protein 1 (AP1) by phosphorylation. AP1-p may then also activate proinflammatory genes that play a central role in RA pathogenesis [65] (Fig. 3B).

Upregulation of ER stress signalling plays a critical role in several diseases such as RA which is activated in immune and somatic cells such as FLS, B cells, myocytes, myeloid cells and chondrocytes [99]. FLS demonstrate invasive features, like tumour cells, which lead to increased synovial proliferation, joint destruction and persistent inflammation [100]. Activation of ERS key factors

such as GRP78, IRE1, XBP1s, and eIF2-P have been reported in macrophages and synovial tissues of patients with RA [101]. The upregulation of GRP78, IRE1 and XBP1s have also been observed in PBMC of patients with RA [88]. Indeed, the inflammatory responses in RA can induce the ERS pathway, and UPR activation may also stimulate the production of inflammatory cytokines, such as TNF [98,102,103].

One of the key markers in ERS pathway is GRP78, which plays a critical role in the pathogenesis of RA [100]. GRP78 overexpression not only occurs in the synovium of patients but may also act as autoantibody in sera of patients with RA [101,104]. Anti-citrullinated BiP (citBiP) antibodies are also detectable in the serum, FLS and macrophages of these patients. In addition to their role in disease causation, extracellular GRP78 acts as an immunoregulatory marker and induces a number of immunosuppressive cytokines such as IL-4 and IL-10 to prevent RA development [105]. Moreover, BiP is also involved in other cellular functions such as antibody production, T cell proliferation and proinflammatory cytokine generation, and therefore has potential to be targeted or induced in the treatment of RA [106]. Increased levels of IRE1 in FLS and macrophages of patients with RA induce the production of TLR-mediated proinflammatory cytokines. The increased expression of XBP1s in PBMC and FLS of active RA patients in contrast to healthy controls or those in remission has also been observed and its activation shown to be mediated through TLR2/4 signalling, leading to IL-6 and TNF secretion. However, in active RA, the other ERS mediators such as ERN1, HSPA5, or SYN1 are decreased, suggesting the IRE1-XBP1s pathway act in a TLR-dependent manner without any impact on ERS signalling [107]. The increased level of PERK-eIF2 present in synovial tissues and macrophages of patients with RA can activate the NF- κ B pathway and increase TNF production [101]. In addition, GADD34, a downstream target of the PERK/eIF2a/ATF4 pathway, is elevated in PBMC of RA patients and impacts on proinflammatory cytokine production [108].

ATF6 is another branch of UPR that is increased in the macrophages of patients with RA. ATF6 may stimulate the inflammatory response and TNF may induce the cleavage of ATF6 in FLS; however, activation of ATF6 may be inhibited by use of proteasome inhibition or autophagic functions [109].

6.5.1 ER Stress-Apoptosis Pathway

One of the major mechanisms of RA pathogenesis is dysregulated apoptosis mechanisms that cause abnormal expansion of FLS, the most common cell type contributing to joint destruction in RA at the pannus–cartilage junction [110]. Although apoptosis is activated by two major types of signal, either an extrinsic death receptor or an intrinsic mitochondrial death receptor, other models of cell death have also been proposed, including T-cell-mediated cyto-

toxicity and perforin-granzyme signalling [111,112]. In the exogenous route, following the binding of Fas ligand (FasL, CD95L) to Fas (CD95) receptors, various proteins are activated that result in upregulation of both initiation and activator caspases, with subsequent activation of proteins involved in apoptosis. The endogenous pathway is activated by a range of intracellular signals such as increased oxidative stress or DNA damaging agents. These factors impact mitochondrial outer membrane permeabilisation (MOMP) and are associated with release of the apoptotic effectors. Apoptosis-dependent mitochondria are regulated by various members of the BCL-2 family, which have both anti- and pro-apoptotic functions [110,113,114].

Under severe ERS, when the cells are no longer able to establish and maintain homeostasis, the three aforementioned UPR pathways trigger apoptosis pathway, via different mechanisms. For example, permanent activation of PERK leads to increased levels of CHOP transcription factor which regulate the expression of many anti-apoptotic and pro-apoptotic genes, including genes encoding the BCL2-family proteins. Furthermore, PERK protein enhances the expression of GADD34 that can dephosphorylate eIF2 α to induce the translation of proteins involved in ER stress-induced apoptosis [84].

CHOP transcription factor, which is regulated by ATF4, induces BIM (a pro-apoptotic member of the BCL-2 family) expression to trigger cell death [115]. Indeed, ATF4 and CHOP increase the levels of reactive oxygen species (ROS) production in stressed cells leading to cell death [116]. On the other hand, severe ERS may cause hyperphosphorylation of IRE1 α and decreased GRP-78 levels, such that unfolded proteins are upregulated, in order to sensitise the cells to apoptosis. In this situation, XBP1 splicing is decreased, which may lead to the switch from the pro-survival UPR function to the task of pro-apoptosis [117]. Furthermore, RIDD activity is reduced in order to activate the apoptotic programmers [118]. Ca²⁺ is released from the ER and taken up by the mitochondria, which triggers cytochrome c release to further promote apoptosis [119]. As RA progresses, the FLS becomes apoptosis-resistant, leading to synovial hyperplasia with release of a range of proinflammatory cytokines. During RA flares, CD147 levels significantly increase in FLS and promote TNF-induced apoptosis by increasing intracellular Ca²⁺ levels via activation of the NF- κ B pathway [120]. Increased levels of synoviolin, an ER-resident E3 ubiquitin ligase, are present in the synovial cells of patients with RA, resulting in a ‘hyper-ERAD’ state, which is a critical factor in the pathogenesis of RA [121]. Synoviolin levels are mainly contributed to by the ERAD system and increased synoviolin leads to the apoptosis-resistant synovial cells characteristic of RA [122].

6.5.2 ER Stress-Autophagy Pathway

Autophagy is a self-eating process activated to remove the impaired organelles, unusual and inactive proteins, or other intracellular substances through lysosomal degradation. This recycling process is crucial for optimal cell life and function [122]. The various molecular mechanisms of autophagy are initiated by formation of autophagosome vacuoles to digest unwanted intracellular contents, which then fuse with lysosomes to form autophagolysosomes for selective degradation of specific cargos, such as organelles and proteins, with reuse of the primary materials. Each step in the process involves regulatory proteins, and mutation in these autophagy genes (ATGs) lead to failure of autophagic mechanisms at different steps in the pathway [123].

It has been reported that the expression levels of Beclin1, ATG5, and LC3, as autophagy-related proteins, are all raised in the synovial tissue of patients with RA [124]. It has also been documented that anti-TNF therapy and use of IL-6R inhibition bring about a reduction in the autophagic response [125]. Furthermore, the use of hydroxychloroquine and chloroquine as autophagy inhibitors drugs, in treating patients with RA, may decrease activation of immune systems and reduce cytokine production [111,126]. 3-MA, as an autophagy inhibitor, also reduces citrullinated peptide levels in RA with induction of apoptosis pathways in RA-FLS compared to OA-FLS [127]. Autophagy can competitively inhibit apoptosis in proliferating FLS in RA [128]; the FLS induce autophagosome formation and enhance the expression of HMGB1 and Beclin-1 [129]. Autophagy is also activated under severe ERS to eliminate any unwanted or misfolded proteins by a mechanism referred to as ERphagy [77]. In addition, there is crosstalk between the ER stress and autophagy mechanisms. IRE1 activation leads to an increase in tumour necrosis factor receptor-associated factor 2 (TRAF2) and consequently apoptosis occurs via kinase-1 (ASK1) and c-JNK signalling. This process mediates the phosphorylation of Bcl-XL/Bcl-2 and enhances Beclin-1 expression [130]. PERK activation also regulates autophagy through ATF4 and CHOP transcription factors, which enhance the expression of autophagy-related proteins, including ATG-12 and ATG5 which bind to ATG16L to induce autophagosome formation [131,132]. In RA, proinflammatory cytokines such as TNF are activated to induce autophagy via conversion of LC3-I to LC3-II [133,134] which leads to apoptosis and drug resistance. Hence, autophagy inhibition, or the use of an anti-TNF treatment may aid in reactivation and restoration of apoptosis in RA [129,135]. For instance, suppression of mTOR signalling in autophagy, in combination with MTX, is used to treat RA [136] and has shown a better response in RA patients than MTX alone; these findings suggest that autophagy modulators could be added to treatment protocols to increase therapeutic effectiveness. However, due to the dual role of autophagy in the pathogenesis of RA, autophagy induction in the early steps of RA may be used to

increase apoptosis [129]. Autophagy exercises a dual role of both cytoprotection and cell death induction in RA-FLS. ER homeostasis is dependent on autophagy activation and a functioning ERAD pathway. Indeed, autophagy activation is correlated with CHOP reduction, with protection of FLS from apoptosis. The use of taspargin as an ERS inducer leads to independent autophagic cell death, whereas the use of MG132 as a proteasome inhibitor induces the cytoprotective effects of autophagy [102].

6.5.3 ER Stress-Redox Signalling

Reactive oxygen species (ROS), including superoxide anion, hydroxyl radical, hydroperoxyl, and hydrogen peroxide are all produced in the cells via endogenous or exogenous sources due to impaired oxygen reduction capacity [136]. Endogenous sources of ROS may include products of metabolism in intracellular organelles such as mitochondria, ER, peroxisomes, and macrophages [137]. Exogenous ROS may be derived from radiation, the use of certain drugs, smoking, and infection [138,139]. The intracellular accumulation of ROS leads to oxidative stress [139], which may play a central role in the development of different autoimmune diseases, such as RA [140].

Changes in intracellular Ca^{2+} regulate the activity of reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite, mainly produced by nitric oxide synthases in mitochondria, and NADPH oxidases (Nox). These RNS may prove to be harmful when reacting with ROS to form highly reactive molecules, such as peroxynitrites, that generate new epitopes to break immune tolerance [141]. Increased levels of ROS have been observed in the serum and synovial fluids of patients with RA [142].

Furthermore, correct folding of proteins is completely dependent on redox status, as increased oxidative stress impairs the ER folding process which, in turn, may lead to the accumulation of unfolded proteins, UPR activation, ROS production, more inflammation, and ultimately apoptosis activation [143]. The use of ERS inhibitors or antioxidants reduces the levels of ROS and proinflammatory cytokines which, in turn, leads to inactivation of the UPR markers [137,144].

The ER and mitochondria engage in crosstalk to maintain the cellular homeostasis through redox signalling, Ca^{2+} transfer, cell death and inflammation [145]. The contact sites in mitochondria are known as mitochondria associated membranes (MAMs) and are rich in ER chaperones [146]. Calcium signalling plays a key role in ER-mitochondria communication and regulates multiple processes involved in cell metabolism, proliferation, differentiation, gene activation and cell death [147]. Under conditions of ER stress (ERS), the connections between these two organelles increases, favouring Ca^{2+} uptake by the mitochondria and increased production of ATP in order to adapt to the condition of stress [146]. Disulfide bond formation in the ER is a significant source of ROS and dysregulated

disulfide bond formation leads to ROS accumulation and oxidative stress. Protein folding is also dependent on an appropriate redox balance, and increased oxidative stress can further impair the ER folding capacity and contribute to the accumulation of misfolded proteins and oxidation, thereby aggravating the UPR response, ROS generation, inflammation, and triggering cell death [137].

6.5.4 The UPR and Inflammatory Signalling Pathways

There is significant crosstalk between the ERS and inflammatory pathways, as ERS induces the production of the cytokines, with triggering all three ER stress sensor proteins, IRE1, PERK and ATF6, which combine to inducing an inflammatory response, through activation of NF- κ B with a resultant increase in proinflammatory cytokine secretion [56]. In normal situations, NF- κ B binds to the inhibitor kappa B (I κ B) and remains inactive. However, in the presence of inflammation and pattern recognition receptors (PRR) signalling, I κ B is phosphorylated and then degraded. NF- κ B is subsequently released and moves to the nucleus where it can induce the expression of proinflammatory cytokines [57]. This, in turn, promotes NF- κ B activation and increased proinflammatory cytokine levels. Thus, the IRE1-XBP1 axis promotes NF- κ B-mediated inflammation via interaction with TRAF2 to activate and degrade I κ B kinase. The IRE1-TRAF2 axis also induces JNK and active transcription factor activator protein 1 (AP-1) to induce an inflammatory response (Fig. 3B). eIF2a phosphorylation in the PERK axis decrease the translation of all proteins such, as I κ B; hence, the level of free NF- κ B is sufficiently raised to stimulate inflammatory cytokine production. Activated ATF6 can directly induce acute phase response genes [148]. ATF6 interacts with phosphatidylinositol 3-kinase (PI3K)-dependent AKT phosphorylation, which is an upstream inhibitor of autophagy, and also can be regulated by IRE1 and PERK [149]. Furthermore, during ERS, TLRs are activated to induce proinflammatory cytokines. In this regard, TLR2 and TLR4 both induce XBP1 activation in FLS of patients with RA to promote both IL-6 and TNF expression. TNF itself also induces XBP1s in FLS, to trigger a feedback loop for the purpose of keeping the FLS active [106]. UPR are involved in cell differentiation in many secretory cell types in the immune system. For instance, although, in the differentiation of monocytes into macrophages, increased levels of BiP and XBP1s were observed, the specific signalling pathway of UPR was not improved. Overexpression of BiP also leads to apoptosis resistance of macrophages rather than of monocytes [150].

6.6 Other Pathways Involved in RA Pathogenesis

6.6.1 The VEGF Signalling Pathway

One of the key features of cancer and inflammation is angiogenesis or activated vascular development, which is involved in tissue growth, dysregulated tissue perfusion, and increased responses to normal or pathological stimuli. The vascular endothelial growth factor (VEGF) super-

family and their receptors (VEGFRs) are important factors for the regulation of vascular permeability [151]. VEGF family members are found in both transmembrane and soluble format. VEGF and its receptors play the major role in synovial tissue pathology in adult RA. Seven isoforms of VEGF have been identified, including PlGF and snake venom VEGF. This growth factor is considered to be the most essential of the three different VEGF isoforms with at least 9 subtypes due to RNA splicing. Each subtype is believed to have a unique function in the development of blood vessels and arteries. VEGF signalling involves binding to tyrosine kinase receptors—VEGFR1 and VEGFR2. VEGFR2 promotes vascular growth, while VEGFR1 acts as a negative regulator during embryogenesis and inflammatory responses. VEGFR3, a receptor for lymphatic growth factors, regulates vascular and lymphatic endothelial function in development. Loss-of-function VEGFR3 variants have been found in lymphedema [64].

The presence of angiogenesis in the synovial lining is essential for RA pannus formation which relies on new blood vessels for growth. The initial discovery of highly vascularized pannus led to intensive investigations into angiogenesis factors in RA, with VEGF being the most potent in both adult RA and juvenile chronic arthritis (JCA) [152]. VEGF causes increased vessel permeability, which plays a critical role in development of chronic oedema and swelling in RA. It also generates chondrocyte and osteolytic fragments found in joint effusions. Although VEGF levels were higher in patients with inflammatory arthritis, there were no significant differences between the types of arthritis studied [64].

6.6.2 The SYK Signalling Pathway in RA

Spleen tyrosine kinase (SYK) is a key part of B-cell receptor (BCR) signalling and phosphorylated SYK levels are significantly higher in RA patient's peripheral blood B-cells [153]. Bruton's tyrosine kinase (BTK) is a crucial molecule in the Tec family of non-receptor tyrosine kinases. It is present in all hematopoietic cells except T-cells and NK cells [154]. BTK links BCR signalling, chemokine receptor and TLR signalling, and has important roles in B-cell regulation [154]. BTK is activated by SYK or PI3K in an antigen-dependent BCR signalling context and regulates B-cell survival and proliferation [155]. BTK can also interact with multiple molecules to promote antibody secretion, cell proliferation, generation of pro-inflammatory cytokines and regulation of B-cell migration [156]. B-cell dysregulation in RA leads to production of autoantibodies and cytokines, which promote disease progression. Moreover, increased levels of phosphorylated BTK in B cells of RA patients, lead to FLS proliferation. In RA patients who are RF positive, phosphorylated BTK is correlated with RF levels [157]. Since BTK regulates osteoclast proliferation and differentiation, this could be a viable target in treating RA [158].

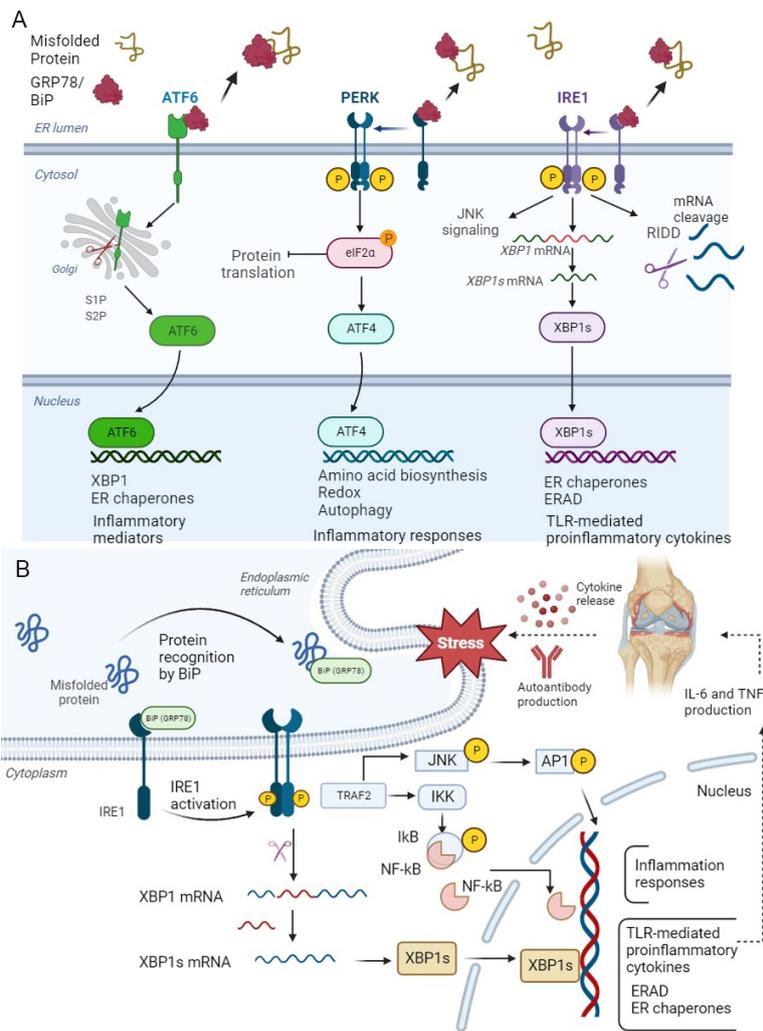


Fig. 3. Unfolded Protein Response (UPR) and IRE1 α branch Signalling Pathways. (A) UPR activation. Under endoplasmic reticulum (ER) stress, GRP78/BiP is dissociated from three arms of UPR and attach to misfolded proteins. IRE1 α is oligomerised and autophosphorylated, leading to activation of RNase domain which can splice the XBP1 mRNA (XBP1s). XBP1s factor moves to the nucleus, acts as transcription factor and increases the expression levels of ERAD, ER chaperone genes, and the genes controlling inflammatory responses. The RNase domain of IRE1 α also degrades selected mRNA in a regulated IRE1-dependent decay (RIDD) pathway. Activation of PERK leads to phosphorylation and inactivation of eIF2 α , which leads to a decrease in global protein translation. However, phosphorylated eIF2 α can increase the transcription factor ATF4, which can stimulate the genes involved in amino acid metabolism, autophagy and inflammatory responses. Following the dissociation of ATF6 from BiP, it is transferred to the Golgi where it is cleaved by the site-1 and site-2 membrane proteases (S1P and S2P) into an active form. Cleaved ATF6 transfers to nucleus and increases the expression of genes such as ER chaperones and inflammatory responses. (B) IRE1 α and RA. In RA proinflammatory cytokines and autoantibodies lead to ER stress response. Of the three arms of the UPR, IRE1 α is the most important and is involved in inflammatory responses. Under ER stress, BiP is dissociated from IRE1 α and attached to misfolded protein. Oligomerization and autophosphorylation of IRE1 α leads to activation of the RNase domain to splice the mRNA of XBP1. The spliced format (XBP1s) acts as transcription factor and stimulate the genes involved in TLR-mediated proinflammatory cytokine release, such as IL-6 and TNF, which can promote the level of inflammation in patients with RA. On the other hand, IRE1 α can interact with TRAF2 and activate I κ B kinase (IKK). Activated IKK leads to the dissociation of I κ B from NF- κ B. The NF- κ B then translocates to the nucleus and induces a number of proinflammatory genes that regulate cytokines, chemokines, and matrix-degrading enzymes. The IRE1 α -TRAF2 complex also activates c-JNK and consequently activates activator protein 1 (AP1) by phosphorylation. AP1-p then also activates proinflammatory genes. Retrieved from <https://app.biorender.com/biorender-templates>.

6.6.3 The Wnt Signaling Pathway in RA

Cancer and embryonic development, as well as RA, are characterised by abnormal activation of the Wingless/Integrated (Wnt) signalling pathway. This pathway plays a central role in synovial inflammation and bone metabolism in RA [159]. Two Wnt pathways, classical and non-classical, are involved in secretion of proteins. The classical Wnt family is involved in secreted frizzled family transmembrane receptor protein Dishevelled (Dsh), glycogen synthesis kinase 3 (GSK3), β -catenin, APC, Axin, and TCF/LEF family transcriptional regulators [160]; while the non-classical Wnt pathway including Wnt-Frizzled/PCP signal conduction, by regulating calcium within the cell. Dsh signals through the Rac1 axis and Daam1-RhoA axis. NAV2 is a part of the neuro-guiding protein family [161], which is involved in development of the nervous system and also plays a role in RA FLS inflammatory responses by activating Wnt/ β -catenin signalling. It has been demonstrated that blocking NAV2 can restrict the extent of inflammation in RA and is considered as a candidate for inclusion in the treatment armamentarium for RA [162].

6.6.4 The Notch Signalling Pathway in RA

Notch genes code for receptors that regulate cell development in organisms and affect cell processes like proliferation, differentiation, apoptosis, and boundary formation. The Notch pathway mainly consists of receptors, ligands, and regulatory molecules. Mammals have 4 receptors and 5 ligands. Notch signalling involves the interaction between adjacent cells via the Notch ligand and receptor. Cleavage of Notch protein releases NICD into the cytoplasm, which then enters the nucleus to form the NICD/CSL complex and activate its target genes. Notch signalling induces inflammation in RA by stimulating various cells to produce proinflammatory cytokines, while its inhibition of this pathway impairs Th17 cell differentiation [163]. For example, Notch-1 binds to IL-17 and ROR- γ T promoters to regulate Th17 differentiation, while Notch-3 is crucial to T-cell differentiation and is upregulated in FLS. Blocking Notch-3 reduces the extent of inflammation and joint injury in experimental inflammatory arthritis [164].

6.6.5 Metabolites and RA Pathogenesis

RA is characterized by synovial hyperplasia and increased proliferation of immune cells, including mainly B-cells, T-cells, dendritic cells, and macrophages which all play critical roles in RA pathogenesis. To adapt to these changes, the cells undergo metabolic reprogramming which creates a vicious cycle that further accelerates the degree of inflammation in the affected joint. Herein, the relationship between metabolites and RA is discussed (Fig. 4).

6.6.5.1 Lactate (Lactic Acid). Hypoxia is a basic metabolic change that occurs in almost all inflammatory disease states including RA. Hypoxia has been shown to cause mitochondrial dysfunction [165]; therefore, to meet the energy demands, cells must undergo a metabolic shift to anaerobic glycolysis. Decreased glucose levels increase intracellular lactate. The lactate transporters MCT-4 and SLC5a12 are upregulated to facilitate the transfer of lactate from the cytosol to the interstitial fluid within the synovial joint [166]. The increased lactate levels within the interstitial space activates quiescent synovial fibroblasts which lead to secretion of growth factors and consequently accelerates proliferation and migration of these cells as well as driving angiogenesis. Biniecka *et al.* (2016) [165] demonstrated an increase in the secretion of basic fibroblast growth factor (bFGF) as well as increased invasion of RA synovial fibroblasts, when cultured with increased lactic acid levels. This hostile microenvironment facilitates the recruitment of leukocytes that aggravate the inflammation within the synovial joint, leading to formation of a pannus which is the characteristic clinical manifestation of RA.

Lactic acidosis is also associated with alteration of the p53 suppression gene which increases cell survival by conferring survival advantages, such as evasion of apoptosis, and increased proliferation. This may result in a consequent exacerbation of inflammation. In fact, levels of lactate dehydrogenase (LDH) enzyme, which is responsible for the conversion of pyruvate into lactate are reported to be elevated within the synovium of patients with RA [167].

6.6.5.2 Succinate. Previous work has shown the significance of measuring levels of the tricarboxylic acid (TCA) metabolite succinate in the diagnosis of RA. Kim *et al.* (2014) [168] found succinate to be a potential biomarker for RA ($p < 0.0001$, AUC = 1 in RA patients compared to non-RA patients). In fact, succinate was shown to be elevated in macrophages [169]. It activates HIF1 α which consequently promotes interleukin 1- β (IL- β) production through activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome. Furthermore, HIF- α increases vascular endothelial growth factor (VEGF) which, in turn, promotes angiogenesis within the inflamed joint, recruiting more inflammatory cells [166].

6.6.5.3 Citrate. Citrate plays a crucial role in cell metabolism and is the key regulator of various metabolic reactions that are the essential sources of energy production [166]. Citrate has also been shown to be a modulator of inflammation and provides a source of NADPH which is necessary to produce both reactive oxygen and nitrite species. Citrate can be converted to the anti-inflammatory metabolite, itaconate, via the enzyme aconitate decarboxylase 1 (ACOD1) [166,170]. Citrate was found to be elevated in the synovial fluid of patients with RA, indicating its poten-

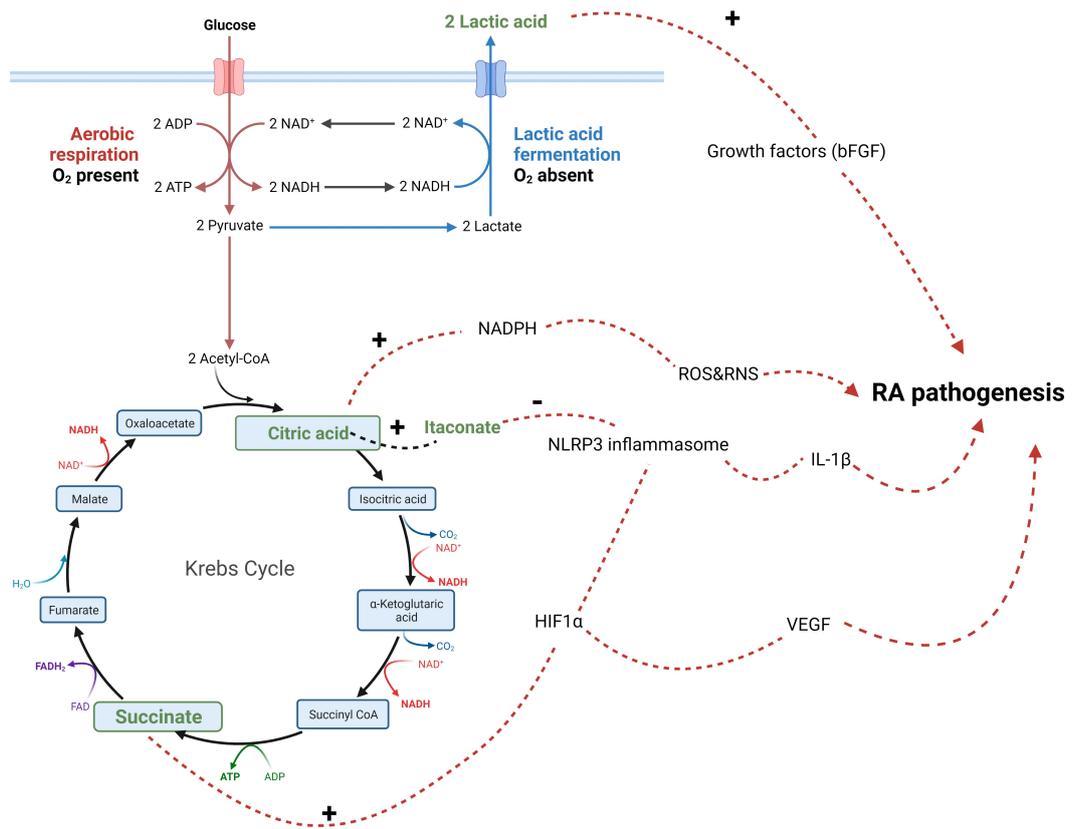


Fig. 4. Metabolites and RA pathogenesis. Lactic acid (lactate) stimulates the release of growth factors from synovial fibroblasts. Citric acid (citrate) provides a source for NADPH, a co factor for production of reactive oxygen and nitrogen species (ROS and RNS). Citrate can be converted into itaconate, which inhibits activation of NLRP3 inflammasome. Succinate activates HIF1 α which leads to production of vascular endothelial growth factor (VEGF) and activation of NLRP3. Adapted from “Lactic Acid Fermentation” and “Krebs Cycle”, by BioRender.com (2023). Retrieved from <https://app.biorender.com/biorender-templates>.

tial use as a biomarker of RA [168]. However, the precise mechanism(s) on how it affects the progression of RA remain to be explored.

6.6.5.4 Itaconate. Itaconate is a vital cell metabolite that has recently emerged in the progression and diagnosis of RA. It is well known to possess anti-inflammatory properties [171–173] and activates the key transcription factor Nrf2 which downstream induces gene expression of antioxidant and anti-inflammatory molecules [171]. Itaconate has also been shown to inhibit the pro-inflammatory complex protein NLRP3 inflammasome [174] NLRP3 initiates a cascade of events leading to production of inflammatory cytokines such as IL-1 β [175]. There is clear evidence to support the involvement of IL-1 β in the progression of RA [176–178] and, similarly, the NLRP3 inflammasome is activated in CD4 T cells of RA patients [179]. The anti-inflammatory properties are further described by Aso *et al.* (2023) [180] who showed that itaconate suppressed TH17 while promoting T regulatory differentia-

tion in CD4⁺ T cells. In this study, the authors propose a novel mechanism independent of Nrf2 activation. Itaconate treatment suppressed glycolysis and oxidation phosphorylation which resulted in a decreased S-adenosyl-L-methionine/S-adenosylhomocysteine ratio (SAM/HG ratio) and decreased 2 hydroxyglutarate (2-HG). Consequently, leading to altered chromatin accessibility that inhibited IL-17A and promoted FOXP3 expression [180]. A recent study also showed that Itaconate inhibits M2 polarization through inhibition of the JAK1/STAT6 pathway [181]. It is of interest that JAK inhibitors are currently being used to treat RA for patients resistant to csDMARDs [182,183] Finally, Daly *et al.* (2020) [184] reported an increase in itaconate levels for patients on csDMARDs. Altogether, these studies present compelling evidence of the immunomodulatory effects of itaconate in RA and, therefore, this remains an area of active investigation.

6.6.6 Programmed Cell Death in RA

Peresolimab is a monoclonal antibody that stimulates human programmed cell death protein 1 (PD-1), which is predominantly expressed on activated T cells. Peresolimab has the capacity to induce physiological immune inhibitory pathways that restore immune homeostasis, but the exact function of PD-1 in Tregs remains unclear at this point. In a phase 2 trial of peresolimab in RA, the peresolimab arm of this study was shown to meet the primary endpoint for efficacy, with similar rates of adverse events between the drug and placebo arms [185]. It is quite possible that this drug, marketed by Eli Lilly, will be of significant therapeutic benefit in treating patients with RA, based on previous experience with the use of immune-checkpoint inhibitors in cancer immunotherapy [186].

7. Responses to Treatment and Drug Resistance in RA

Despite the wide-ranging targets and armamentarium of therapy available to treat RA, there are still patients with RA who unfortunately never quite achieve remission or even low disease activity states, the so-called difficult-to-treat RA [187]. These include drug resistant RA, recognised as refractory RA [188]. Drug resistance in RA is likely to be multifactorial, and can stem from several causes, such as the development of neutralising antibodies against biologics, alterations in drug metabolism, or shifts in the underlying disease mechanism [189]. There is also the recognition that the age of onset of RA can affect the response to treatment, with more drug resistance with increasing age of onset [190].

Personalised medicine and the ability to stratify and predict patients' response to therapy may reduce the drug resistance in RA. Recently, it has been shown that an increase in dendritic cell precursors can predict the prognosis of RA [191]; such findings provide opportunities for novel targets for precision RA therapies. With improved understanding of the pathogenesis of RA comes advancement in the various facets of precision medicine in RA. Multidrug resistance is one of the most common reasons for refractory RA [192], but the ability to identify the right drug for a particular patient with a RA is somewhat lagging behind [193]. Real world registry studies may have a place in helping to close the gap in RA precision medicine, as we understand how patients respond to different therapies, particularly as patients with resistant RA are likely to have cycled through a number of different biologics and targeted synthetic DMARDs (tsDMARDs) [194].

8. Conclusions

Delineation of the intracellular signalling pathways activated in RA and development of novel targeted therapies are essential steps to improving patient outcome in this complex disease.

As befits the heterogeneity of drug resistant RA, there is a complex myriad of potential targets for therapy [195, 196]. Therefore, the knowledge gained in discovering the intracellular signalling pathways in RA will complement precision medicine strategies in providing difficult-to-treat RA, particularly those exhibiting drug resistance and may also offer the hope of achieving disease remission, or, at least, a less active disease state.

Author Contributions

MR and MFM conceived the study and retrieved the literature; MPK, JL and ALT retrieved the literature. All the authors were involved in writing the paper, and all authors read and approved the final manuscript. All authors have participated sufficiently in the work and are accountable for all aspects of the work. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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