Review

Targeting fibroblast-like synoviocytes with CDK4/6 inhibitors as a novel, non-immunosuppressive treatment strategy for rheumatoid arthritis

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ABSTRACT

Despite advances in rheumatoid arthritis (RA) therapy, a substantial proportion of patients fail to achieve remission with current targeted immunomodulators. The synovial joint and its cells mediate a central role in pathobiology of RA. Within the synovial joint, fibroblast-like synoviocytes (FLSs) are a stromal mesenchymal cell population that have been implicated in the initiation and development of rheumatoid arthritis and may contribute to poor response and treatment resistance. Multiple lines of evidence including genetics, in vitro experiments and animal arthritis model studies implicate the cell-cycle proteins, cyclin dependent kinases (CDKs), CDK4 and CDK6, as key regulators of FLS activity in RA suggesting them as a potential therapeutic target.

CDK4/6 inhibition is an established therapeutic strategy in breast cancer, and importantly, translational and clinical data observed in oncology patients demonstrate decreasing aromatase inhibitor-induced arthralgia, or ameliorating symptoms of concomitant RA by treatment with currently approved oncology CDK4/6 inhibitors. However, a narrow therapeutic index paired with the risk of myelosuppression and QT-interval prolongation precludes their use in non-oncology indications such as RA. Therefore, next generation CDK4/6 inhibitors with improved therapeutic index have been designed. A recently published phase 1b study of TCK-276 in RA patients, indicated promising signs of early clinical efficacy and an absence of class-related side effects.

This review summarises the molecular biology of CDK4/6, its role in RA pathobiology and discusses how therapeutic CDK4/6 inhibition could form a novel therapeutic class addressing unmet need in the treatment of RA.

Introduction

Despite advances in rheumatoid arthritis (RA) therapy a substantial proportion of patients fail to reach remission (1). Disease-modifying anti-rheumatic drugs (DMARDs) include conventional synthetic DMARDs (csDMARDs), biological DMARDs (bDMARDs) and targeted synthetic DMARDs (tsDMARDs). Briefly, European Alliance of Associations for Rheumatology guidelines advise that initial treatment should include a csDMARD, methotrexate, in combination with glucocorticosteroids. Depending on prognostic factors and response to first line therapy, guidelines recommend changing/adding another csDMARDs (such as leflunomide or sulfasalazine), or introducing a bDMARD or tsDMARD. The current repertoire of approved immune-targeting therapies (2) includes four classes of bDMARDs and one class of tsDMARDs, Janus kinase (JAK) inhibitors, which have shown strong clinical efficacy in clinical trials and in real-world studies. However, a notable percentage of patients experience continued symptoms whilst receiving bDMARDs. Incomplete clinical response affects ~40% of patients on bDMARD monotherapy and a subset of between 5–20% of patients fail to respond to all current medications. Overall, it is estimated that >50% of patients fail to achieve remission or maintain low disease activity (3).

In these patients, cycling between available bDMARDs is an option but often yields diminishing clinical success. An alternative strategy is the use of JAK inhibitors whose efficacy is unaffected by

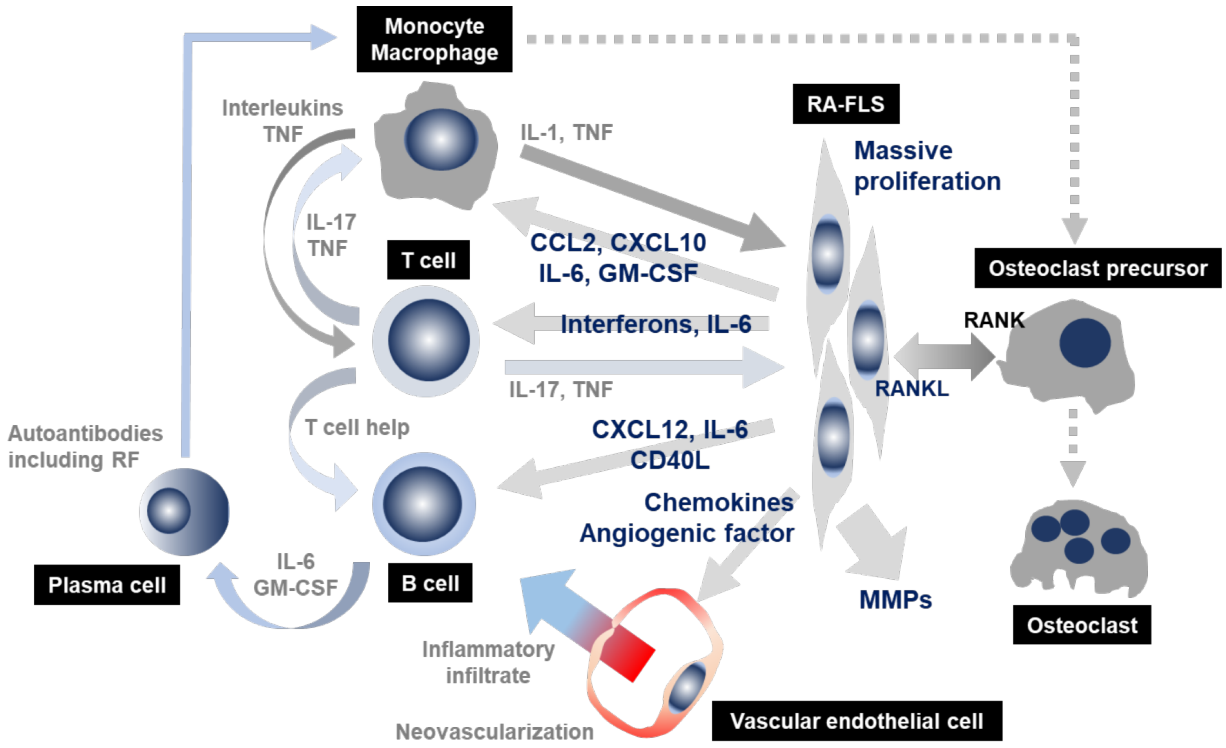

Fig. 1. RA-FLS in the pathophysiology of RA.
RA-FLS play a central role in the pathobiology of RA synovitis. The number of FLS is considerably increased in RA synovial tissue. Functional contributions of RA-FLS are joint destruction (overproduction of MMPs, stimulating the differentiation and activation of osteoclasts) and synovial tissue inflammation (facilitating the influx of pro-inflammatory cells, secreting a variety of pro-inflammatory factors including angiogenic factors, chemokines and inflammatory cytokines). Interactions between RA-FLS, T cells and B cells in the rheumatoid joint participate in amplifying the autoimmune response. Current treatment approaches focus on altering the adaptive or innate immune responses mainly mediated by B and T cells (not specifically addressing aberrant RA-FLS biology).
CCL2: CC-chemokine ligand 2; CD40L: CD40 ligand; CXCL10: CXC-chemokine ligand 10; CXCL12: CXC-chemokine ligand 12; FLS: fibroblast-like synoviocyte; GM-CSF: granulocyte/monocyte colony stimulating factor; IL: interleukin; MMP: matrix metalloproteinase; RANK: receptor activator of nuclear factor κB; RANKL: RANK ligand; RF: rheumatoid factor; TNF: tumour necrosis factor.

previous treatment failure (4, 5). JAK inhibitors are however associated with a higher risk of specific cardiovascular, thrombotic, and infectious side effects compared to bDMARDs (6). Finally, intensifying immunosuppression by combining immunotherapies, *e.g.* anti-tumour necrosis factor (TNF) with anti-interleukin (IL)-1 or co-stimulation blockade, has yielded marginal efficacy benefit whilst significantly increasing adverse events (7, 8).

Accordingly, there remains significant unmet medical need for adjunctive treatment options. Ideally, adjunctive treatment options would act via orthogonal, non-immunosuppressive mechanisms that can be more safely combined with targeted immunomodulation. An attractive potential target for novel therapies is synovial cells. Within the synovial joint, fibroblast-like synoviocytes (FLSs) are a specialised stromal mesenchymal cell population which has been extensively implicated in the RA disease process.

FLS in the pathobiology of RA

FLSs populate the intimal lining of the synovial tissue and maintain the normal structural and dynamic integrity of healthy diarthrodial joints by controlling the composition of synovial fluid and extracellular matrix (ECM) of the joint lining (9-13). In RA, the structural and functional features of the synovial tissue are profoundly altered with both massive infiltration of the synovial sub-lining by inflammatory cells and synovial hyperplasia (Fig. 1). The role of fibroblasts in immune response and inflammatory diseases is summarised in a recent review by Zou *et al*. (14). Thus, FLSs are extensively implicated in the pathological process of RA through cartilage/bone destruction, production of pro-inflammatory mediators and activation of the autoimmune response.

Synovial hyperplasia

The number of FLSs in RA (RA-FLSs) in the rheumatoid joint increases considerably with advancing disease course (15, 16), driven by a combination of decreased apoptosis and increased proliferation resulting in the formation of a hyperplastic tissue mass known as a pannus (17, 18). RA-FLSs proliferation is driven by the activation of the RAS-RAF-MEK-ERK mitogenic pathway that leads to DNA replication through negative regulation of the retinoblastoma tumour suppressor (RB)-E2F transcription pathway (19-21) (Fig.2A).

E2F is upregulated in RA-FLS in RA compared to healthy controls, and its expression is further increased in response to TNF (22). E2F is associated with pathological progression and exacerbation of inflammatory phenotypes in RA-FLS (23).

These facts demonstrate that the number of RA-FLS in the rheumatoid joint

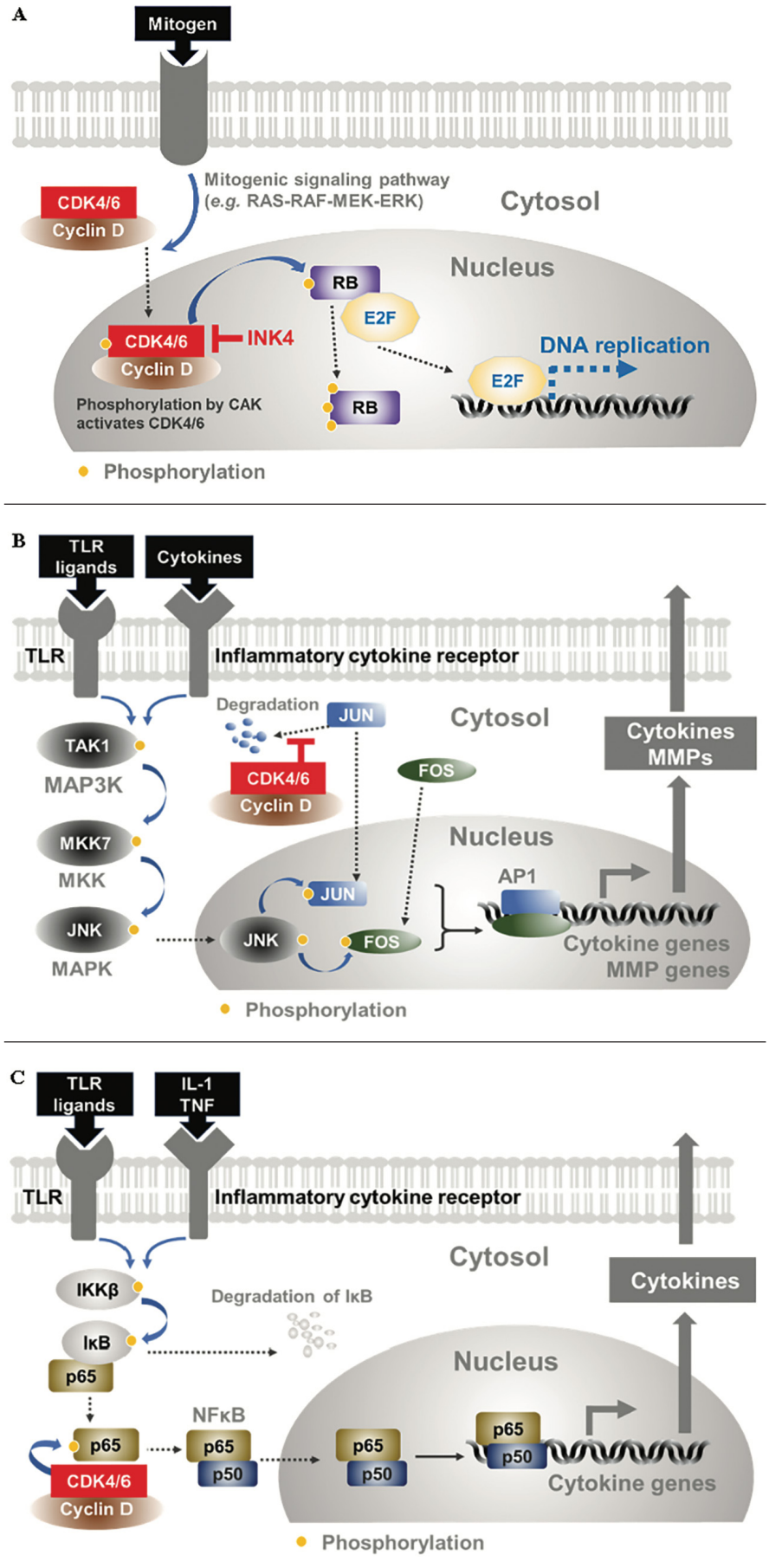

Fig. 2. Molecular mechanism in RA-FLS pathogenesis.

A: RB-E2F pathway. Cyclin dependent kinase 4/6 (CDK4/6) have a crucial role in cellular proliferation. CDK4/6 form a complex with cyclin D and regulate entry to the cell cycle, progression of G1 phase and initiation of DNA replication through the RB-E2F pathway. CDK4/6 phosphorylate RB and inactivate RB's nuclear function which in turn causes E2F activation. INK4 is an intracellular protein that inhibits CDK4/6.

B: MAPK pathway. Signal transduction involves the activation of three levels of kinases: the first tier includes MAPK kinase kinase (MAP3K); the second tier includes MAPK kinase (MKK); and the third tier comprises MAPK including JNK. Once activated through phosphorylation by MKK, MAPK can migrate from the cytosol to the nucleus where it phosphorylates and activates a variety of important transcription factors responsible for cell differentiation, proliferation, migration and survival. CDK4/6 protect JUN from degradation and allow the induction of AP1 transcriptional activity. Inhibition of CDK4/6 enhances ubiquitin dependent degradation of JUN which in turn leads to suppression of genes regulated by AP1.

C: NFκB pathway. Signalling through the NFκB pathway requires the inhibitor of NFκB kinase subunit β (IKKβ) in the cytosol. Activation of IKKβ leads to phosphorylation of proteins of the inhibitor of NFκB (IκB) family. IκB proteins form complexes with cytosolic subunits of NFκB, maintaining them in an inactive state. After phosphorylation by IKKβ, IκBs are degraded by the proteasome, leaving NFκB free to migrate to the nucleus and initiate gene transcription. CDK4/6 interact with NFκB subunit p65 and promote NFκB activity by phosphorylating p65. Inhibition of CDK4/6 suppresses p65 phosphorylation which in turn leads to suppression of NFκB-induced gene expression.

CAK: CDK activating complex, which phosphorylates T172 (CDK4) or T177 (CDK6) which in turn fully activation of CDK4/6; ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MEK: MAPK ERK kinase; NFκB: nuclear factor κB; RB: retinoblastoma tumour suppressor; TAK1: tumour growth factor-β-activated kinase 1; TLR: toll-like receptor

increases considerably with advancing disease, and increased RA-FLS contribute to the transformation of normal synovial tissue into a hyperplastic tissue. Proliferation of RA-FLS is regulated by RB-E2F pathway, and upregulation of E2F is associated with pathological progression.

Contribution to joint destruction

RA-FLSs acquire functional changes that contribute to the disease process. The principal pathobiological feature of RA-FLS is their direct contribution to destruction of cartilage and periarticular bone. At the pannus–cartilage interface of the rheumatoid joint, overproduction of matrix metalloproteinases (MMPs) and cathepsins by RA-FLS damages the collagen-rich structures of the joint tissue (24-29). Overproduction of cartilage-degrading enzymes is driven by the mitogen-activated protein kinase (MAPK) pathway and mediated by the c-Jun N-terminal kinase (JNK)-transcription factor AP1 axis (30, 31). RA-FLSs retain a semi-autonomous destructive and invasive phenotype when transplanted into SCID mice, acting independently of lymphocytes and lymphocyte-driven inflammation (32).

Besides cartilage destruction, RA-FLSs are also directly involved in driving bone erosion through the expression of receptor activator of nuclear factor κB ligand (RANKL) which stimulates the differentiation and activation of osteoclasts (33, 34). Accordingly, synovial hyperplasia driven by RA-FLS is associated with a 12-fold higher risk of progressive cartilage/bone destruction in RA as well as with a poor clinical prognosis (35).

Contribution to synovial tissue inflammation

A second important feature of RA-FLS is their ability to drive inflammation in the rheumatoid joint in an autocrine and paracrine manner via resident stromal cells and infiltrating immune cells in synovial tissue (Fig. 1). Pro-inflammatory mediators produced include angiogenic factors (15, 36, 37), chemokines (15), and inflammatory cytokines such as IL-6 that will lead to further activation of both tissue cells and infiltrating immune cells.

The production of pro-inflammatory factors by RA-FLS is principally driven by the MAPK and nuclear factor κB (NFκB) pathways. The MAPK pathway (Fig. 2B) is the most extensively researched in RA-FLS. RA-FLSs harbour several molecular alterations that promote activation of the MAPK pathway (19-21, 38). One of the important MAPK-activated mediators in RA-FLS is the transcription factor AP1, a heterodimer of phosphorylated JUN and FOS subunits. AP1 is upregulated in RA (39). JNK is the primary physiological kinase that phosphorylates and activates JUN. Knockdown studies have shown that the upstream kinase dual-specificity MAPK kinase 7 (MKK7) is the most important MAPK kinase for activation of JNK in RA-FLS, and the MKK7-JNK-AP1 axis is primarily activated by tumour growth factor (TGF)-β-activated kinase 1 (TAK1) in RA-FLS (40).

A second important signalling pathway in RA-FLS is the NFκB pathway, which is a major regulator of pro-inflammatory cytokine production and is activated by IL-1, TNF and toll-like receptor (TLR) signalling (Fig. 2C). Inhibiting the NFκB pathway by inhibiting the inhibitor of NFκB kinase subunit β (IKKβ) reduces the activation and survival of RA-FLS (41).

These facts demonstrate that the ability of RA-FLS to produce a variety or pro-inflammatory factors is mediated by the MAPK and NFκB pathways, sustaining and augmenting inflammation in the synovial tissue.

Modulation of autoimmune response in synovial tissue

A third and emerging pathobiological feature of RA-FLS is their ability to attract, activate and support (auto)immune cells, for instance contributing to T cell survival and activation (42-44), presenting arthritogenic peptides to T cells (45, 46). Similarly, RA-FLS can support B cell survival (47-49), as well as differentiation and activation of memory B cells (50). Moreover, RA-FLS can be the source of B cell autoantigens (51).

Collectively, these data support the concept that interactions between RA-FLSs and T cells or B cells in the rheumatoid joint participate in amplifying the autoimmune response (46, 52, 53).

FLS in refractory RA

Multi-omics analyses from a biopsy-based randomised clinical trial in RA (R4RA study) revealed that the gene profiles of RA synovial tissue from patients refractory to bDMARDs, tocilizumab and rituximab, demonstrated enriched stromal/fibroblast gene signatures, while those who respond are associated with inflammatory or humoral immune response gene signatures (54, 55). Another study using cell-type abundance phenotypes (CTAPs) analysis, revealed that fibroblast dominant CTAPs are associated with refractory disease characteristics, while immune cell rich CTAPs decrease after the treatment with bDMARDs or tsDMARDs (56). RA-FLS may also contribute to pain sensing in patients with limited synovial inflammation and persistent joint pain (57).

Collectively, these phenotypic, functional and translational data indicate that RA-FLSs are not merely passive responders to inflammation but play a much more central role in the pathobiology of RA synovitis (Fig. 1).

RA-FLS as a potential target

D'Orazio *et al*. (58) highlight that recent findings support the active role of RA-FLSs in RA and help to explain the mechanisms underlying RA pathogenesis. Furthermore, these observations support the rational of RA-FLSs as potential cellular targets for novel therapies at various stages in the development and progression of RA.

Few RA-FLS-specific candidate targets have been identified and evaluated to date, besides cadherin-11 and integrin-α9. Despite promising preclinical data, clinical development of anti-cadherin-11 (RG6125) and anti-integrin-α9 (ASP5094) therapies was discontinued owing to a lack of obvious efficacy in phase 2 trials. This could potentially explained by a suboptimal PK/PD profile impairing antibody diffusion to the target synovial tissue.

Recently, early clinical data using roscovitine (Seliciclib) to target RA-FLS were published (TRAFIC trial) (59, 60). Roscovitine is a broad-spectrum

cyclin dependent kinase (CDK) inhibitor of CDK1, CDK2, CDK5, CDK7, and CDK9 (61, 62). Non-clinical data showed a reduction in passively induced arthritis in mice (63). The Phase 1b trial explored once daily roscovitine in addition to TNF blockade in patients with active RA. The patients demonstrated dose limiting toxicities at 400 mg. In the nine patients who completed the four weeks study period, the DAS28-CRP score decreased by ~1.5 points (60). This trial highlights the potential role of CDKs as a molecular target in RA-FLS and underlines the importance of a broad therapeutic index.

In summary, RA-FLSs have been proposed as a potential non-immune therapeutic target. However, developing drugs targeting RA-FLS remains in its infancy. Few RA-FLS-specific candidate targets have, to-date, been identified besides cadherin-11 and integrin-α9. Recent clinical investigation on roscovitine highlights CDKs as molecular targets in RA-FLS.

Table I. Publications summarising involvement of CDKs (other than CDK4/6) in the pathobiology of RA-FLS.

CDKs	Association with disease	Inhibiting CDKs and their effects on disease
CDK1	Elevated in blood, positive correlations with ACPA (100)	• THRIL inhibits FLS proliferation; CDK1 is one of its downstream targets (101).
CDK2	NA	• MiR-34a-3p inhibits transcriptional and post-transcriptional expression of CDK2 (102). • MiR-124a inhibits proliferation of RA-FLS through targeting 3'-UTR region of CDK2 mRNA (103).
CDK7	NA	• BS-181 inhibits IL-1β, IL-6, IL-8, and RANKL in RA-FLS (104,105).
CDK8	STAT1 is a CDK8 target gene (106).	• Deficiency decreases osteoclastogenesis (107).
CDK9	NA	• Targeting CDK9 with flavopiridol inhibits the expression of the RANKL-induced osteoclast-related genes (108). • Inhibition of CDK9 shows anti-arthritic effect in CIA mice (109).

THRIL: a long non-coding RNA, downregulated in RA and inhibits proliferation of RA-FLS (101).
BS-181: CDK7 inhibitor, and it inhibits the inhibitor of nuclear factor κB kinase subunit β phosphorylation (105).
ACPA: anti-cyclic citrullinated peptide; CDK: cyclin dependent kinase; CIA: collagen-induced arthritis; FLS: fibroblast-like synoviocyte; NA: not applicable; RANKL: nuclear factor κB ligand; STAT1: signal transducer and activator of transcription 1.

CDK and evidence for CDK4/6 inhibition in RA

From the late 19th through the middle of the 20th century, seminal discoveries were made about cellular mitotic division. These findings have played a foundational role in our understanding of key events during cell division, a sequence we now call the 'cell cycle'. The cell cycle comprises four phases, G1, S, G2 and M. From the 1990s onwards, advances in cell cycle biology further advanced our knowledge of how cell cycle transitions are regulated which led to the discovery of cyclins and CDKs (64-68). Among the CDK family, CDK4/6 form a complex with cyclin D regulating entry to the cell cycle and initiation and progression of G1. G1/S transition, progression of S phase, or G2 phase are regulated by CDK2-cyclin E, CDK2-cyclin A, or CDK1-cyclin A, respectively. G2/M transition and progression of M phase is regulated by CDK1-cyclin B. The most accepted model of G1 progression suggests that CDK4/6 activity is sufficient to induce RB hyperphosphorylation and E2F activation, while CDK2-cyclin E or A maintains hyperphosphorylation in S phase (69).

Table I summarises the role of CDKs (excluding CDK4/6) in RA-FLS. CDK1, CDK2, CDK7, CDK8, or CDK9 have also been considered as molecular target candidates. First generation CDK inhibitors, *i.e.* pan-CDK inhibitors active across multiple CDKs listed above (*e.g.* CDK1, CDK2) were associated with a range of side effects including nausea, diarrhoea, and myelosuppression which limited the prolonged dosing with these inhibitors (69).

Although a variety of CDKs are reported to be potentially important in the pathobiology of RA driven by RA-FLS, CDK4/6 are considered the most promising based on genetics, mode of action, *in vitro* experiments, and animal arthritis model studies. In addition, importantly, translational observations in patients treated with currently approved oncology CDK4/6 inhibitors identify CDK4/6 as a relevant molecular target for RA synovitis.

Genetic evidence supporting a role for CDK4/6 in RA

As much as 24% of disease susceptibility in RA is related to single-nucleotide polymorphisms (SNPs) in germline genes that act on RA-FLS (70). *CDK4/6* have been identified as disease-susceptibility genes in RA. A genome-wide association study (GWAS) in 100,000 subjects of European and Asian ancestries (29,880 RA cases), evaluated 10 million SNPs (71). This study identified *CDK4* and *CDK6* as 'candidate biological risk RA genes' with gene risk scores comparable with those of *TNF*, *IL-6*, and *CTLA4*.

Snir *et al*. investigated the association of previously discovered RA loci, including *CDK6*, with disease-specific autoantibody responses in RA patients stratified by HLA alleles (72). Their analysis showed allelic correlation between *CDK6* SNP and anti-cyclic citrullinated peptide in non-carriers of HLA-DRB1*04. *CDK4* was not investigated in this study.

Ge *et al*. conducted fine mapping of more than 100 genetic risk loci in RA identified in GWAS, by integrating DNA architecture, 3D chromatin interactions, DNA accessibility, and gene expression in RA-FLS (70). The analysis showed that RA-FLS activation observed in RA joints was clearly reflected in the predicted function of the identified RA-FLS specific regulatory variants, many of which were previously associated with RA pathogenesis and are connected by functional

networks. They found that *CDK6* SNPs play an important role modulating the behaviour of RA-FLS but do not have a meaningful role in immune cells. This analysis also demonstrated that a significant proportion of the 73 European ancestry non-HLA RA association signals contain disease-associated variants located within active regulatory DNA elements in RA-FLS.

In summary, genomic data implicate *CDK6* and *CDK4* as potential risk alleles for RA. Germline SNPs affecting the CDK6 gene fall in a promoter region and could in principle lead to a gain of function, but the precise genetic mechanism is unclear and requires further experimental investigation.

Mode of action of CDK4/6 in RA pathogenesis

Mechanistic studies have demonstrated that CDK4/6 regulate several pathways that play an important role in the pathogenesis of RA (Fig. 2A-C). CDK4/6 form a complex with cyclin D, which phosphorylates RB and promotes E2F activity, inducing a transition into S phase, cell cycle progression, and proliferation (Fig. 2A). CDK4/6 inhibition attenuates RB phosphorylation preserving its suppressive effect on targets of the E2F transcription factor (73-75). INK4, an intracellular protein that inhibits CDK4/6 (76), is shown to suppress cellular proliferation of RA-FLS (77).

Besides controlling RA-FLS proliferation by regulating the RB-E2F pathway, recent evidence indicates that CDK4/6 also regulate inflammatory pathways, including the MAPK pathway. Activation of cells – including RA-FLS – by TLR ligands and cytokines leads to a signalling cascade resulting in JUN- and FOS-dependent stimulation of AP1 transcriptional activity, which leads to transcription of MMPs and other RA-associated genes (Fig. 2B). Hosoya *et al*. showed that when CDK4/6 is inhibited, auto-amplification of JUN and FOS is impaired via enhanced ubiquitin dependent degradation of JUN protein. Accordingly, the CDK4/6 inhibitor, palbociclib suppressed MMP1 and MMP3 by regulating AP1 transcriptional activity (30). Using transcriptomic analysis, Hosoya and colleagues confirmed that inhibition of CDK4/6 results in the selective suppression of genes that are regulated by JUN and FOS.

CDK4/6 inhibition also indirectly regulates inflammatory pathways in RA fibroblasts. NFκB regulates inflammation and induces the expression of a number of pro-inflammatory cytokines and mediators in RA-FLS. CDK6 binds to and phosphorylates p65 promoting NFκB activity (78) (Fig. 2C). Handschick *et al*. demonstrated that the translocation and activation of NFκB subunit p65 is CDK6-dependent (79). Increased CDK6 expression causes NFκB p65 phosphorylation and NFκB-induced gene expression. NFκB is activated upon stimulation with pro-inflammatory cytokines, such as IL-1 or TNF-α. RNAi-mediated knockdown of CDK6 suppressed several IL-1-induced genes including IL-8 and IL-6 (79). In this investigation, the authors could not show direct evidence of CDK4 interaction with p65, however they also suggested that CDK4 employs alternative mechanisms for regulation of gene expression. A recent study demonstrated that the activation of the NFκB pathway by the stimulator of interferon genes (STING), a DNA sensor, is also CDK4/6 dependent (80).

In summary, these mechanistic data indicate that CDK4/6 is not only important for cell cycle progression and proliferation but also directly impacts critical pro-inflammatory pathways such as the MAPK and NFκB pathways.

In vitro experiments

Intracellular proteins that inhibit CDK (CDKIs) include two families, INK4 and Cip/Kip (77). Taniguchi *et al*. analysed the expression of $p15^{INK4b}$, $p16^{INK4a}$, $p21^{Cip1}$, and $p27^{Kip1}$ in mature synovial cells. Normal synovial tissue expressed $p27^{Kip1}$ but not $p15^{INK4b}$, $p16^{INK4a}$, or $p21^{Cip1}$. Interestingly, expression of $p16^{INK4a}$ was induced when RA-FLSs were growth inhibited *in vitro*. In addition, transfection and overexpression of the $p16^{INK4a}$ gene suppressed RA-FLS proliferation in response to TNF-α and IL-1 (77). These data demonstrate that, 1) $p16^{INK4a}$ inducibility is characteristic of RA-FLS and 2) RA-FLS unlike non-rheumatoid synovial or other fibroblasts express $p16^{INK4a}$ when their growth is inhibited in culture.

Nasu *et al*. showed similar findings with $p16^{INK4a}$ in RA-FLS. In addition, they forced expression of $p21^{Cip1}$, which inhibits the CDK2-cyclin E/A complex, and showed that both $p16^{INK4a}$ and $p21^{Cip1}$ suppress proliferation of RA-FLS *in vitro*. They note that induction of $p16^{INK4a}$ under growth inhibitory conditions is characteristic of rheumatoid synovial fibroblasts (in contrast to $p21^{Cip1}$) and therefore agonism of $p16^{INK4a}$ may be a preferable pharmacological target.

Hosoya *et al*. reported that transfection of CDK4 and cyclin D genes in RA-FLS stimulated with TNF-α and IL-1β potentiates the production of MMP1 and MMP3 (30). Importantly, the inhibition of CDK4/6 (via overexpression of the $p16^{INK4a}$ gene) reduced both the transcriptional and translational levels of MMP3 and CC-chemokine ligand 2 (CCL2) in RA-FLS (81). Nonomura *et al*. demonstrated that $p18^{INK4c}$, which is the other INK4 family protein inhibiting CDK4/6, suppressed RA-FLS proliferation as well as production of MMP3 and CCL2.

Small-molecule inhibition of CDK4/6 has also been investigated. Sekine *et al*. reported a study of flavopiridol (pan-CDK inhibitor, including CDK1, CDK2, CDK4/6, and CDK7) which inhibited cellular proliferation in a concentration-dependent manner in human RA-FLS and FLS from collagen-induced arthritis (CIA) mice. G1 cell cycle arrest was observed following flavopiridol treatment, and apoptotic death was not seen (82).

These facts demonstrate that *in vitro* experiments have identified CDK4/6 as critical regulators of the aggressive phenotype of RA-FLS.

Animal arthritis model studies

Adenoviral gene therapy with the $p16^{INK4a}$ gene efficiently inhibits adjuvant arthritis (AA) (77). Suppression of synovial tissue hypertrophy and inhibition of joint swelling was observed in these rats. Histology revealed that mononuclear cell infiltration and pannus formation were inhibited, cartilage and proteoglycan were well-preserved, and synovial tissue thickening was effectively suppressed. Whilst CDK4/6

Table II. Side effects/risks of oncology CDK4/6 inhibitors.

Events	Descriptions			
Myelosuppression	• CDK4/6 drive proliferation of blood cell precursors and its inhibition can impact cell counts resulting in neutropenia, lymphopenia, anaemia (110).			
Neutropenia	• All oncology CDK4/6 inhibitors have warnings for neutropenia (FDA labels*).			
	• Neutropenia caused by palbociclib was reported in patients with breast cancer, prompting dose reduction or treatment discontinuation (Phase 1 study) (111).			
	• PALOMA-2 phase 3 study of palbociclib, 80% of patients developed neutropenia (all grades), including 56% grade 3-4 neutropenia (112).			
	• Real-world data of palbociclib, ribociclib, and abemaciclib (retrospective analysis on patients with metastatic breast cancer, analysed toxicity profile from 205 patients) showed grade 2 or higher neutropenia with all 3 agents (113).			
		Palbociclib	Ribociclib	Abemaciclib
	Grade 2 neutropenia	25.0%	18.8%	20.8%
	Grade 3 neutropenia	42.5%	46.9%	13.2%
	Grade 4 neutropenia	1.7%	0.0%	0.0%
	• Decrease in neutrophil count was observed 1 to 2 weeks after the start of therapy (113).			
Lymphopenia infections	• Palbociclib-related lymphopenia may contribute the risk of serious infections and reactivation of latent viruses (114).			
	• Meta-analysis demonstrated that the use of oncology CDK 4/6 inhibitors is associated with an elevated risk of infections (115).			
QT-interval prolongation	• QT interval prolongation-risk has been reported in ribociclib (116, 117).			

* Available from:
Palbociclib: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/207103s008lbl.pdf
Ribociclib: https://www.accessdata.fda.gov/drugsatfda_docs/label/2022/209092s013,209935s021lbl.pdf
Abemaciclib: https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/208716s006s007s008lbl.pdf
CDK: cyclin dependent kinase.

activity was not directly assessed in this study, since $p16^{INK4a}$ overexpression negatively regulates CDK4/6 activity in RA-FLS, it was concluded that this type of gene therapy was mediating its effects via CDK4 and CDK6.

Confirming these data, forced expression of $p16^{INK4a}$ suppressed arthritis in the mouse CIA model, both in a preventive and in a therapeutic setting (83). The effects included suppression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, demonstrating that CDK4/6 inhibition by $p16^{INK4a}$ overexpression not only prevents synovial proliferation but also ameliorates the pro-inflammatory milieu in affected joints.

Treatment of CIA mice with flavopiridol also causes a dose-dependent reduction in arthritis in both early and late disease (82). Interestingly, Sekine and colleagues also investigated the efficacy by flavopiridol in K/BxN serum-induced arthritis model induced in lymphocyte-deficient $RAG2^{-/-}$ mice, confirming that the therapeutic efficacy of flavopiridol is not dependent on effects on T or B cells.

Palbociclb, a CDK4/6 inhibitor approved for treatment of human breast cancer was evaluated as a monotherapy and in combination with bDMARD (anti-TNF-α or anti-IL-6) in CIA mice (84). Palbociclib monotherapy significantly decreased the arthritis score and combination with bDMARDs further improved histological and radiographic scores. Interestingly, targeting CDK4/6 resulted in a strong improvement in arthritis without impacting autoantibodies or T cell responses, thus supporting the concept that CDK4/6 principally acts via an orthogonal non-immunological mechanism.

These facts demonstrate that *in vivo* studies have confirmed the role of CDK4/6 as critical regulators of inflammatory arthritis. Furthermore, targeting RA-FLS through inhibition of CDK4/6 has emerged as an attractive and viable novel strategy to treat arthritis.

Translational and clinical data

Due to the major role of CDKs in regulating the cell cycle, CDKs have become an attractive therapeutic target in neoplastic diseases, and several agents targeting CDKs have been developed in the oncology field since the 1990s. To date, five CDK4/6 inhibitors have been approved for the treatment of HR^+/$HER2^-$ advanced breast cancer: palbociclib (85), ribociclib (86), abemaciclib (87), trilaciclib (88), and dalpiciclib (89). None of these 'oncology' CDK4/6 inhibitors have been tested in patients with RA in a clinical trial setting. However, several publications involving palbociclib, ribociclib as well as abemaciclib have generated translational data suggesting therapeutic effect of inhibiting CDK4/6 in patients with RA.

Andrikopoulou and colleagues reported that an oncology CDK4/6 inhibitor plays an important role in the suppression of aromatase inhibitor (AI)-induced arthralgia in patients with breast cancer. Musculoskeletal symptoms, including arthralgia and arthritis, are common side effects of AI in breast cancer patients (90), mediated through the induction of an autoimmune process that affects the joints, sharing similarity to RA (91-93). AIs promote the infiltration of the synovial tissue by T cells and the cytokine-induced activation of monocytes, macrophages, and FLSs in the joint. Data from a systemic review of randomised controlled trials evaluating the co-administration of AIs and oncology CDK4/6 inhibitors in oncology patients provide evidence that the arthralgia rates reported in these patients co-treated with CDK4/6 inhibitors tend to be lower compared with patients receiving AI monotherapy alone (73-75).

A case report by Murakami *et al*. described that administration of palbociclib to a patient with metastatic breast cancer and concomitant RA was associated with a decrease in RA symptoms and

reduced serum CRP and MMP3 concentrations (94). Whilst these data are from a single patient, they suggest that palbociclib could act via CDK4/6 inhibition to ameliorate symptoms of RA.

Whilst translational data suggest that CDK4/6 inhibitors may have applications in RA, the side effect profile of currently approved oncology CDK4/6 inhibitors precludes their use in non-oncology indications. Table II summarises the side effects of current CDK4/6 inhibitors which include myelosuppression – neutropenia, lymphopenia, risk of severe infections and electro cardiogram changes, specifically QT-interval prolongation. The previously mentioned pan-CDK inhibitor roscovitine was tested in a phase 1b trial in RA patients and dose-limiting toxicities were observed at 400 mg including gastrointestinal symptoms (constipation, nausea, vomiting) and liver enzyme abnormalities (74). An improved safety profile is thus a prerequisite for the potential use of CDK4/6 inhibitors in non-oncology indications such as RA.

These facts imply that translational observations in patients treated with currently approved oncology CDK4/6 inhibitors support the role of CDK4/6 as relevant molecular targets in treating RA synovitis. However, the type, frequency and severity of common side effects associated with oncology CDK4/6 inhibitors preclude their use in RA.

Next generation selective CDK4/6 inhibitor

Considering the therapeutic potential but narrow therapeutic range of the currently approved CDK4/6 inhibitors, focused drug discovery campaigns were initiated to find selective and potent CDK4/6 inhibitors with an improved therapeutic index. TCK-276, a novel small-molecule selective inhibitor to CDK4/6, possesses a high selectivity, potent inhibitory activity, and inhibits proliferation and MMP secretion in RA synovial fibroblasts *in vitro* (95).

In mouse CIA, mouse collagen antibody induced arthritis (CAIA), and rat AA, TCK-276 dose-dependently inhibited clinical arthritis, histological bone destruction, and serum MMP3 levels (95). The combination of TCK-276 with anti-TNF-α further suppressed arthritic scores and MMP3 levels compared to either therapy alone. The *in vivo* efficacy of TCK-276 was confirmed in mouse CIA as well as CAIA studies (96). The authors demonstrated suppressed MMP3 expression in joints in addition to decreased serum MMP3 levels which was associated with amelioration of arthritis scores. Importantly, TCK-276 did not reduce type II collagen IgG levels, suggesting that TCK-276 has no impact on the adaptive immune system.

Improved safety profile and therapeutic index

Myelosuppression was not observed at therapeutically effective doses of TCK-276 (95). In addition, non-clinical animal toxicity studies of TCK-276 demonstrated a favourable therapeutic index. Safety margins were eight-fold and two-to-five-fold compared to the myelosuppressive dose level in rodents and monkeys, respectively (97). With regards to cardiac function, TCK-276 had no inhibitory action in hERG transfected CHO cells (97) and proarrhythmia study in human iPS-derived cardiomyocytes revealed a very low risk of TCK-276 on cardiac function suggesting that TCK-276 has a "clean" cardiovascular safety profile (95). In addition, no safety concerns were demonstrated in monkey cardiovascular telemetry studies (95).

Clinical experience with the next generation CDK4/6 inhibitor in RA

Recently, Tasaki *et al*. reported a phase 1b placebo controlled multiple ascending dose study of TCK-276 dosed once daily for seven days conducted in 32 patients with active RA (97). Whilst the phase 1b study of TCK-276 was primarily designed to assess safety/tolerability and PK of TCK-276, it revealed a preliminary signal of clinical efficacy. Improvement in DAS28-CRP scores and ACR20 responses was noted as early as seven days after treatment initiation. This trend was seen in both the 75 mg/day and 175 mg/day dose groups, whereas no improvement was seen in the placebo and the 10 mg/day groups. TCK-276 was well tolerated with no clinically meaningful safety signals. There was no QT prolongation in the studies dose range of 10 mg to 175 mg once daily. Similarly, there were no neutropenia events or alterations in other haematological indices during the seven-day treatment-period or the safety follow up period, a total of 14 days. These clinical data suggest that TCK-276 has a more favourable therapeutic index than approved oncology CDK4/6 inhibitors. Nevertheless, these observations require confirmation over longer duration in a larger clinical trial.

TCK-276 was preferentially taken up by RA-FLS over bone marrow cells (97) in contrast to palbociclib, which is taken up almost equally by both cell types. The differentiated uptake of TCK-276 may underly the observed absence of neutropenia in pre-clinical and clinical studies and thus explain the relatively wide therapeutic index observed.

PK data from TCK-276 showed it has rapid absorption, elimination, and a short half-life (overall $t_{1/2}$ ranged from approximately six to 12 hours after dosing) which appears to be significantly shorter than that of the approved CDK4/6 inhibitors: palbociclib has a reported half-life of 26 to 27 hours (98) and abemaciclib approximately 18 to 24 hours (FDA label). The shorter half-life of TCK-276 may play an important role in avoiding neutropenia and myelosuppression seen with oncology CDK4/6 inhibitors.

Limitations of these phase 1b data include the small study size and the relatively short treatment duration, which should be taken into consideration when interpreting the clinical efficacy signal. Nevertheless, the rapid clinical improvement observed and the absence of haematopoietic and cardiac side effects in this study warrant further clinical evaluation of TCK-276 in a larger group of patients with active RA (97, 99).

In summary, TCK-276, a novel CDK4/6 inhibitor demonstrated a favourable therapeutic index in non-clinical studies compared to approved oncology CDK4/6 inhibitors. TCK-276 demonstrated an absence of clinically significant adverse events, and early signs of clinical efficacy in a phase 1b study in

RA patients. Collectively these non-clinical and clinical data suggest a new potential avenue for synoviocyte-targeted anti-rheumatic therapies in RA.

Conclusion

In summary:

1. Targeting RA-FLS is a promising novel treatment approach. Emerging genetic data, *in vitro* experiments, animal arthritis model studies, and translational observations in patients with breast cancer identify CDK4/6 as a relevant molecular target for RA synovitis.
2. Current CDK4/6 inhibitors approved for use in oncology are associated with prevalent and clinically significant haematopoietic and cardiac adverse events which preclude their use in non-oncological indications such as RA.
3. Next generation selective CDK4/6 inhibitors, as exemplified by TCK-276, were designed to have an improved therapeutic index. Non-clinical animal arthritis model data as well as the phase 1b study in RA patients suggest that TCK-276 does not impact cardiac conduction and does not have the safety signals of other CDK4/6 inhibitors. The rapid clinical improvement observed and the absence of haematopoietic and cardiac side effects in the phase 1b study warrant further clinical evaluation.

Further clinical research will need to confirm mode of action and to assess benefit-risk in different RA subpopulations and in different treatment combinations.

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