Diversity of NF-κB signalling and inflammatory heterogeneity in Rheumatic Autoimmune Disease

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Highlights

1. NF-κB-related genetic variants have been identified in most Systemic Autoimmune Rheumatic Diseases

2. Dysregulated NF-κB is present in most SARDs

3. Cell-specific NF-κB dysregulation may influence the site of inflammation

4. Cell- and pathway-specific NF-κB activity corresponds with RA synovial pathotypes

Abstract

Systemic Autoimmune Rheumatic Diseases, including Rheumatoid Arthritis, Systemic Lupus Erythematosus and Sjogren's syndrome, are characterised by a loss of immune tolerance and chronic inflammation. There is marked heterogeneity in clinical and molecular phenotypes in each condition, and the aetiology of these is unclear. NF-κB is an inducible transcription factor that is critical in the physiological inflammatory response, and which has been implicated in chronic inflammation. Genome wide association studies have linked risk alleles related to the NF-κB pathway to the pathogenesis of multiple Systemic Autoimmune Rheumatic Diseases. This review describes how cell- and pathway-specific NF-κB activation contribute to the spectrum of clinical phenotypes and molecular pathotypes in rheumatic disease. Potential clinical applications are explored, including therapeutic interventions and utilisation of NF-κB as a biomarker of disease subtypes and treatment response.

1. **INTRODUCTION**

1.1 **Importance of NF-κB in Systemic Autoimmune Rheumatic Diseases**

Systemic Autoimmune Rheumatic Diseases (SARDs), including Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), and vasculitis, are characterised by a loss of immune tolerance and chronic inflammation. Each SARD has a spectrum of clinical, serological and in some cases radiological phenotypes which result in a heterogeneous affected population [1]. The pathogenesis of these conditions is clearly multifactorial, with a complex interplay between genetic, epigenetic and environmental factors. One key pro-inflammatory pathway implicated in chronic inflammation is mediated by 'Nuclear factor kappa-light-chain-enhancer of activated B cells' (commonly referred to as NF-κB). NF-κB itself is an inducible transcription factor with a critical role in immune cell differentiation and survival. It contributes to the physiological inflammatory response to a pathogen by upregulating the expression of pro-inflammatory cytokines and chemokines, while also regulating the inflammasome [2, 3]. Although first discovered as the transcription factor stimulating light chain immunoglobulin production in B cells [4], it has since been shown to be constitutively expressed in most immune cells of both lymphoid and myeloid origin.

All SARDs have complex aetiologies with polygenic and likely environmental contributing factors. However, genome-wide association studies (GWAS) have identified multiple risk alleles related to NF-κB, which thus confer a genetic predisposition to developing autoimmune rheumatological conditions. This review aims to summarise current understanding of the significance of NF-κB dysregulation, its role in polarising immune cell differentiation, and in turn its contribution to the heterogeneous molecular and clinical phenotypes observed in SARDs. We will explore whether NF-κB could be utilised as a new biomarker to stratify disease subtypes or treatment response, and its potential as a therapeutic target.

1.2 **Overview of the NF-κB pathway**

NF-κB comprises of 5 structurally related proteins, NF-κB1 (p50), NF-κB2 (p52), Rel-A (p65), Rel-B and c-Rel. These combine to form hetero- or homo-dimers and bind to the DNA promoter region 'κB enhancer' [2]. Transgenic and knockout animal models [5], as well as in vitro human gene silencing

studies [6], have been pivotal in identifying the individual components of these pathways and understanding their contribution to immune dysfunction. NF-κB can be activated by a canonical 'classical' and non-canonical 'alternative' pathway, summarised below and in **Figure 1**.
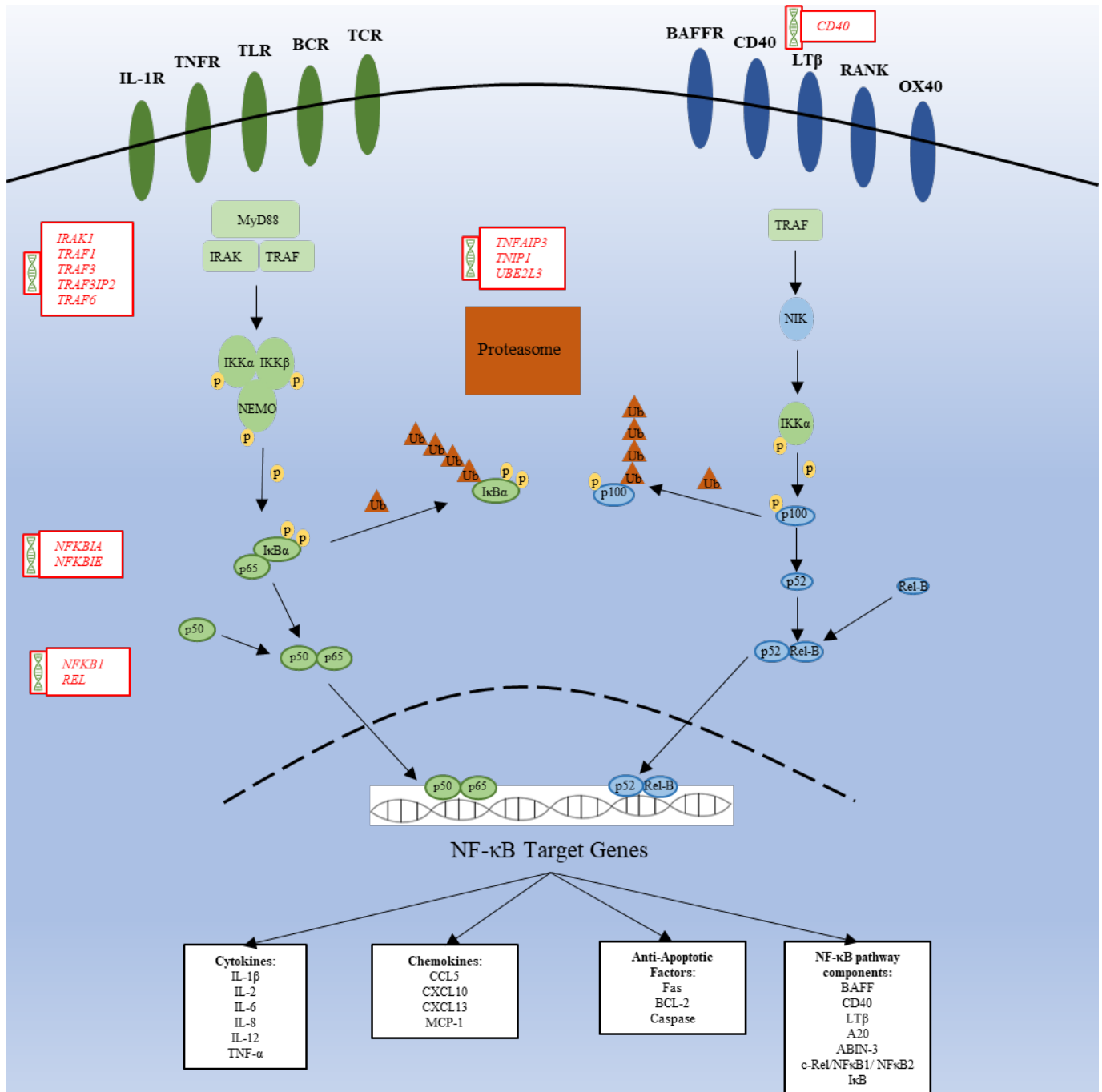

Figure 1: Overview of the canonical and non-canonical NF-κB pathways
The canonical pathway is induced by IL-1 receptor (IL-1R), TNF receptor (TNFR), Toll-Like Receptor (TLRs), B cell receptor (BCR) and T cell receptor (TCR) activation. This stimulates a cascade of phosphorylation resulting in IκB phosphorylation and subsequent proteasomal degradation. This in turn releases p65 to form a heterodimer with p50 and translocate to the nucleus. The non-canonical pathway is induced by B cell activation factor (BAFF), CD40, Lymphotoxin β (LTβ), RANK or OX40 activation and triggers a phosphorylation cascade involving NF-κB inducing kinase (NIK) and IKKα. This results in p100 phosphorylation and release of p52 to form a heterodimer with Rel-B and translocate to the nucleus. NF-κB induces transcription of pro-inflammatory cytokines, chemokines, anti-apoptotic factors, and modulates transcription NF-κB-related components. NF-κB-pathway related genetic polymorphisms are denoted in red in close proximity to their corresponding protein.

Canonical signalling is activated by multiple pro-inflammatory cytokine receptors including TNF receptor (TNFR), Toll-like receptor (TLR) and IL-1 receptor (IL-1R), as well as by immune cell interaction via the T cell receptor (TCR) and B cell receptor (BCR). In contrast, non-canonical NF-κB signalling is activated by cell-cell interactions via cell-surface receptors: Lymphotoxin β receptor (LTβR), BAFF (B cell activating factor, which can also be in soluble form), RANK (Receptor Activation of NF-κB), and costimulatory ligands CD40 and OX40 [3].

Inactive NF-κB is bound to a family of inhibitory proteins IκB, the most researched being IκBα, which anchors p65 in the cytoplasm. p105 and p100, which are precursors of p50 and p52 respectively, are also members of the IκB family, segregating the inactive NF-κB in the cytoplasm. Both IκB and the NF-κB precursors are characterised by C terminal ankyrin repeats, which are targeted for phosphorylation and subsequent ubiquitin-mediated proteasomal degradation, to 'activate' NF-κB by releasing it for nuclear translocation to bind to its DNA promoter site. The IκB kinase (IKK) complex, comprising of IKKα, IKKβ and NEMO (NF-κB essential modulator), is critical in mediating the canonical pathway by site-specific phosphorylation of IκBα to release p65, whilst in the non-canonical pathway NIK (NF-κB inducing kinase) activates IKKα which in turn phosphorylates the C terminus of p100, releasing p52 [2].

The canonical and non-canonical pathways do not function in isolation and there seems to be a considerable degree of crosstalk, particularly in their upstream regulators [7]. The typical canonical heterodimer pair is p50/p65 and non-canonical pair is p52/Rel-B. Lymphotoxin β (LTβ)-mediated non-canonical NF-κB activation is required for the formation of tertiary lymphoid structures [8], which have been identified in the synovium in Rheumatoid Arthritis [9], salivary glands in Sjogren's syndrome [10] and kidneys in a mouse model of lupus nephritis [11]. LTβ deficient mice lose all ability for lymphoid organogenesis. This phenotype is replicated in a mouse model where both p50 and p52 are knocked out, so that neither NF-κB pathway can be activated. However, mice deficient in either p50 or p52 develop defective but not absent lymphoid structures, as there is compensation by alternative NF-κB heterodimer formation into p52/p65 or p50/RelB respectively [12]. In addition, IKKα and NIK are

involved in a negative regulatory loop – NIK phosphorylates IKKα, thereby activating it to subsequently phosphorylate p100, but IKKα also targets NIK for degradation [13], which negatively regulates non-canonical activation. This negative regulatory mechanism is lost if the 'IKKα-binding site' on NIK is mutated and blocks IKKα activation [14]. Gray et al demonstrated that a loss of function mutation of NEMO, which is required for the formation of the IKK complex in activating the canonical pathway, can also result in accumulation of NIK and overexpression of noncanonical p52, via an IKKα independent mechanism [15].

2. Genetic pleiotropy of NF-κB signalling

2.1 Genetics and Genome Wide Association Studies

The advent of genome-wide association studies (GWAS) in the last two decades has accelerated our understanding of the genetic contribution to systemic autoimmune conditions. Over 100 genetic risk loci have been identified for rheumatoid arthritis alone, with **Table 1** summarising single nucleotide polymorphisms (SNPs) identified in genes relating to the NF-κB pathway specifically (odds ratios denote increased risk). Respectively, these SNPs affect the components of NF-κB directly (*REL [16], NFKB1* [17]), inhibitors of NF-κB (*NFKBIE* [18]*, NFKBIA* [18, 19]), receptors (*CD40* [16]), upstream signalling (*IRAK1* [16, 20], *TRAF1* [16], *TRAF3* [21], *TRAF3IP2* [22], *TRAF6* [16]) and the ubiquitination pathway (*UBE2L3* [20, 23], *TNFAIP3* [16, 18-20, 24], *TNIP1* [17, 20, 22, 25]). In rheumatoid arthritis, GWAS have also been performed on patients receiving anti-TNF therapy, with a favourable outcome associated with SNPs in *CD40* [26]*, TNFAIP3* [26], *TRAF1/C5*, *NFKBIB* and *IRAK3* [27]. However, the functional impact of these polymorphisms – and whether they are associated with NF-κB-mediated upregulation of TNF expression – is not yet known.

Likely causal gene at locus	Chromosome location	SNP[1]	Odds Ratio	p value	GWAS reference	Rheumatological Condition
TNFAIP3	6q23	rs6920220	1.2	2.3×10^{-13}	16	Rheumatoid Arthritis
TNFAIP3	6q23	rs6932056	1.82	1.23×10^{-16}	20	Systemic Lupus Erythematosus
TNFAIP3	6q23	rs5029939	1.67	7.75×10^{-9}	24	Sjogren's Syndrome
TNFAIP3	6q23	rs9321623	1.201	5.9×10^{-8}	19	Psoriatic Arthritis
TNFAIP3	6q23	rs610604	1.1	3.25×10^{-3}	19	Psoriatic Arthritis
TNFAIP3	6q23	rs9494893	1.4	4.7×10^{-11}	18	Rheumatoid Arthritis
TNIP1	5q33	rs3792783	1.2	2.42×10^{-12}	17	Systemic sclerosis
TNIP1	5q33	rs76956521	1.5	4.98×10^{-9}	22	Psoriatic Arthritis
TNIP1	5q33	rs10036748	1.32	2.83×10^{-18}	20	Systemic Lupus Erythematosus
TNIP1	5q33	rs6579837	1.43	3.3×10^{-8}	25	Sjogren's Syndrome
TNIP1	5q33	rs7732451	1.34	5.3×10^{-7}	25	Sjogren's Syndrome
IRAK1	Xq28	rs1059702	0.86	8.2×10^{-7}	18	Rheumatoid Arthritis
IRAK1	Xq28	rs13397	1.27	1.2×10^{-12}	16	Rheumatoid Arthritis
IRAK1	Xq28	rs1734787	1.57	2.83×10^{-11}	20	Systemic Lupus Erythematosus
UBE2L3	22q11	rs7444	1.28	1.3×10^{-13}	20	Systemic Lupus Erythematosus
UBE2L3-YDJC	22q11	rs11089637	1.08	2.1×10^{-9}	23	Rheumatoid Arthritis

NFKBIA	14q13	rs12883343	1.22	2.6×10^{-9}	19	Psoriatic Arthritis
NFKBIA	14q13	rs111597524	1.18	1.3×10^{-8}	18	Rheumatoid Arthritis
NFKB1	4q24	rs230534	1.15	5.38×10^{-9}	17	Systemic sclerosis
NFKBIE	6p21	rs1044690	1.19	1.4×10^{-8}	18	Rheumatoid Arthritis
CD40	20q13	rs6032662	0.86	1.4×10^{-9}	16	Rheumatoid Arthritis
MIR146A	5q33	rs2431697	1.25	3.23×10^{-14}	20	Systemic Lupus Erythematosus
REL	2p16	rs34695944	1.13	2.6×10^{-8}	16	Rheumatoid Arthritis
TRAF1	9q33	rs10739580	1.12	1.7×10^{-6}	16	Rheumatoid Arthritis
TRAF3	14q32	rs12148050	0.93	1.04×10^{-3}	21	Systemic Lupus Erythematosus
TRAF3IP2	6q21	rs33980500	1.6	2.65×10^{-16}	22	Psoriatic Arthritis
TRAF6	11p12	rs570676	0.93	2.1×10^{-3}	16	Rheumatoid Arthritis

Table 1: Genetic Variants in NF-κB related genes
Table summarizing single nucleotide polymorphisms in NF-κB pathway related genes identified from Genome Wide association studies in Systemic Autoimmune Rheumatic Diseases.
[1]Single Nucleotide polymorphism

This genetic pleiotropy is perhaps most evident with *TNFAIP3* and *TNIP1*. Although both gene loci are associated with susceptibility to multiple SARDs, associations for specific single nucleotide polymorphisms at both loci differ between SARDs. Interestingly, both genes encode proteins involved in the ubiquitination process (resulting in degradation of the IκB family). In psoriatic disease, *TNFAIP3* is associated with a greater risk of developing psoriatic arthritis rather than cutaneous psoriasis [19], which would suggest that it may play a role in differentiating clinical subtypes.

As SARDs are not monogenic conditions, it is possible that patients may have multiple genetic variants which may interact, conferring a cumulative risk by dysregulating several steps of a given pathway. There is one known human monogenic autoimmune condition which is caused by haploinsufficiency of A20 (the protein of *TNFAIP3*), resulting in increased IκBα degradation and subsequent NF-κB nuclear translocation [28]. These patients develop widespread external and internal mucosal ulceration, including gastrointestinal ulceration, although interestingly with no histological features consistent with vasculitis [29]. This has similarity to Behçet's disease, which is an inflammatory condition resulting in wide-ranging mucosal ulcerations, but notably also vasculitis. However, not all GWAS performed in SARDs have identified NF-κB risk loci – vasculitis and myositis are notable exceptions. This may indicate that aberrations in the NF-κB pathway are not relevant to their pathogenesis. However, it is also possible that these studies were not adequately powered to identify weaker associations, given the low incidence of these rare conditions and substantial heterogeneity – with multiple different autoantibodies associated with distinct clinical phenotypes.

2.2 Functional impact of NF-κB risk alleles

Despite the developments from GWAS, the impact of these genetic variants both in 'healthy' individuals and those who develop SARDs has not been well characterised. Expression (eQTL) and functional quantitative trait loci (fQTL) experiments have been used to investigate links between genetic variants and downstream cis- (local)/trans- (linkage disequilibrium) mRNA expression and functional activity of pathways *in vitro*, respectively. The current assumption is that a SNP relating to a specific protein will lead to altered expression of the protein in favour of activation of an inflammatory process, which has been supported in a few limited studies. A *TNFAIP3* TT/A dinucleotide polymorphism identified in Systemic Lupus Erythematosus (SLE) correlates with reduced *TNFAIP3* mRNA and subsequent suppression of A20 expression, resulting in increased NF-κB activity [30]. This approach has also been used to validate a homozygous SNP (rs4810485) identified at the *CD40* locus in SLE patients, but not reaching GWAS significance. This polymorphism still demonstrated a dose-dependent increase in unstimulated CD40 mRNA and protein expression with the homozygous G/G genotype in monocytes and B cells in vitro, as well as sensitivity to LPS upregulation [31]. The

homozygous T/T *UBE2L3* SNP rs140490 is associated with increased UBE2L3 enzyme expression, and NF-κB activation downstream of CD40L in primary human B cells, and TNF in human monocytes [32]. These functional studies all demonstrate that, as expected, genetic variants do influence the mRNA and protein expression downstream of gene activation. In one study, the homozygous *UBE2L3* risk variant was shown to correlate with plasmablast/ plasma cell expansion in SLE but not healthy volunteers [32], demonstrating a cell- and disease-specific impact of the genetic variant, and indicating that these genetic variants can impact directly on clinical presentation.

2.3 <u>Epigenetic modifications related to NF-κB signalling</u>

Epigenetic modifications, such as histone modification, DNA methylation and expression of microRNA, which are non-coding genetic elements often functioning as negative regulators, can all influence transcription by adjusting access and binding capacity of transcription factors to genes. One of the most investigated microRNAs in SARDs is mir146α, which was identified in an SLE GWAS [20] and found to be suppressed by the *MIR146A* SNP rs2431637 in an eQTL [33]. The interaction between NF-κB and mir146α is complex with each able to regulate the other. NF-κB binds to the mir146α distal promoter to diminish mir146α expression [34], but mir146α can also modulate TRAF6 and IRAK1 expression [35] which acts upstream of NF-κB. Decreased mir146α expression in SLE peripheral blood mononuclear cells (PBMCs) correlates with a greater interferon gene signature [36]. In contrast genetic CRISPR manipulation *in vitro* led to overexpression of mir146α and resulted in suppression of the Type 1 interferon response [34]. This was also replicated *in vivo* with a milder nephritis phenotype in a lupus mouse model treated with mir146α [37], suggesting a further indirect link between NF-κB and Type 1 Interferon response. Conversely, mir146α expression is elevated in synovium and PBMCs from rheumatoid arthritis patients, correlating with inflammatory markers and lack of suppression of TRAF6 and IRAK1 [38] – a finding inconsistent with previous literature. It is not clear however, whether this is derived from an alternative activation of the site independent of NF-κB or epigenetic changes that may prevent modulation of TRAF6/IRAK1.

IKKα is critical in both canonical and non-canonical phosphorylation of inhibitory components of NF-κB (i.e. facilitating NF-κB activation). It also contributes to histone H3 site phosphorylation modifying NF-κB expression [39, 40]. OX40-mediated non-canonical activation of Rel-B in T cells recruits histone methyltransferases to *IL17* gene locus for H3K9 trimethylation, which prevents IL17 expression and Th17 polarisation, demonstrating how NF-κB can contribute to immune cell differentiation [41].

3.   Role of NF-κB in the heterogeneity of Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune condition affecting 1% of the global population and characterised by joint inflammation, pain, progressive damage and disability. RA (along with other SARDs) has a very heterogeneous presentation at the clinical, radiological, synovial and serological (autoantibody status) level [42]. Current clinical practice of stratifying patients is based on serological status (autoantibody positivity for rheumatoid factor and anti-citrullinated cyclic peptide antibody), routine inflammatory markers (C-reactive protein and Erythrocyte sedimentation rate) and composite scores of disease activity that incorporate clinical assessment and inflammatory markers (e.g. DAS28-CRP or DAS28-ESR score) [43]. However, we know that this stratification of patients into subgroups is lacking in sensitivity. Even the composite DAS28-CRP score can be variable due to subjective components which may be influenced by patient perception and/ or assessor expertise (pain score, tender and swollen joint count). For stoical patients with a higher pain threshold this may result in a lower reported disease activity score even in the presence of high degrees of synovial inflammation on ultrasound, and the reverse can also occur.

There is an urgent need for improved stratification markers for disease activity and treatment response, ideally which align with the underlying cause of the heterogeneity in clinical presentations and disease modifying anti-rheumatic drug (DMARD) response. The clinical heterogeneity of rheumatoid arthritis can be demonstrated through the identification of distinct synovial pathotypes [44, 45], transcriptomic diversity [45, 46] and other serological markers such as CXCL13 [47, 48]. These have all been proposed as possible new biomarkers, though none are currently employed in clinical practice. Meanwhile, NF-κB has been most extensively investigated in rheumatoid arthritis, where its cell- and pathway-specific

activation seems to correspond with the three distinct synovial pathotypes identified, and circumstantial evidence supports its contribution to the spectrum of phenotypes and treatment response observed.

NF-κB is upregulated in rheumatoid arthritis-affected synovium, with greater perivascular synovial lining expression [49, 50], and higher DNA binding activity of NF-κB compared to 'healthy' or osteoarthritic synovium [49, 50]. This has been replicated in animal models, with elevated synovial NF-κB DNA binding activity in collagen-induced arthritis [50], and a significant improvement in adjuvant-induced arthritis following loss of function IKKβ gene transfection [51] or proteasomal inhibition [52], which prevent the phosphorylation and ubiquitin degradation of the inhibitory IκB proteins and block NF-κB activation. Thus local synovial inflammation seems to be driven to a large extent by NF-κB. The mechanism of how NF-κB may contribute to RA heterogeneity may be better understood by looking at its activity in each of the synovial pathotypes and how different immune cell recruitment can drive local inflammation.

Synovial biopsy, initially with immunohistochemistry and now with the advances of transcriptomics, has enabled the classification of the synovial pathotype into lympho-myeloid , diffuse myeloid and pauci-immune [45]. The lympho-myeloid pathotype is characterised by synovial macrophage, lymphocyte and plasma cell infiltration with ectopic lymphoid structures, and is a hallmark of a more severe clinical phenotype, associated with local production of ACPA, greater likelihood of progression to erosive disease [44, 53, 54]. The diffuse myeloid pathotype, which is denoted by macrophage infiltration, is associated with a better response to anti-TNF therapy [55]. However, the pauci-immune pathotype is characterised by a fibroblast gene signature and poor immune cell infiltration correlates with anti-TNF, anti-CD20 and anti-IL6R inadequate response [54, 56]. Synovial molecular pathway signatures have enhanced our understanding of synovial pathotypes and show promise to predict markers of treatment non-response and lead a personalised approach to therapy [44, 45, 54].

NIK, which activates non-canonical NF-κB activation has been shown to be critical for lymphoid organogenesis, mediated through BAFF and LTβ receptor (LTβR) activation. A mouse model of NIK

deletion resulted in reduction in B cell populations in lymph nodes and absence of secondary lymph node formation [57]. Elevated levels of NIK and non-canonical NF-κB components are expressed in synovial ELS compared to canonical components, and non-canonical NF-κB upregulated CXCL13 production in endothelial cells [58], thus encouraging immune cell infiltration and ELS formation. Endothelial cell CXCL13 production has also been proposed as the trigger of ELS formation in Sjogren's syndrome [10, 59]. However, Kucharzewska et al discovered that NIK activation in endothelial cells can upregulate the canonical pathway in a p100-independent manner [60]. This finding raises questions as to whether ELS formation is truly solely reliant on non-canonical NF-κB, or also involves the canonical pathway – further definitive investigation is required.

Bone erosions in RA are produced by overactive osteoclast activity, where bone destruction outweighs the degree of bone formation [61]. Several transgenic mouse models have been crucial in understanding the contribution of NF-κB to bone erosion formation. Myeloid-selective A20 knockout resulted in overactive NF-κB, greater TNFα secretion and development of erosive synovitis [62]. A complete ablation of the NF-κB pathway in a double knockout of NF-κB1 (p50) and NF-κB2 (p52) mouse model resulted in absent osteoclast development, which was not observed with individual NF-κB1 or NF-κB2 deficiency [63]. The mechanism of osteoclastogenesis is complex and relies on osteoclast-lymphocyte interactions and RANK ligand activation [64], which is an upstream effector of NF-κB. NIK knockout mice lose the ability to develop antigen-induced synovitis and bone erosions. This process is likely to be T lymphocyte dependent, as transfer of NIK replete T lymphocytes, but not NIK replete B lymphocytes, restored the ability of antigen-induced arthritis to form [65]. This could explain observable trends in clinical practice, where accelerated erosive disease in RA strongly correlates with the lympho-myeloid pathotype [44].

The diffuse myeloid pathotype is associated with macrophage infiltration both in the synovium and synovial lining. Activated elements of canonical NF-κB, nuclear p50 and p65, are upregulated in monocytes and macrophages in RA, but not healthy, synovial lining and related endothelium [66, 67]. These canonical NF-κB elements are absent in synovial ELS [67]. IκBα transfected into RA synovial

macrophages in vitro was found to inhibit spontaneous and LPS-induced TNFα production, by anchoring NF-κB in the cytoplasm [68]. The positive feedback role of TNFα on activating canonical NF-κB, as well as downstream upregulation of TNFα production could account for improved outcomes with anti-TNF therapy in patients with the myeloid pathotype [55]. However, there is evidence to suggest that non-canonical NIK activation in macrophages results in upregulation of co-stimulatory activation of T cells and Th17 polarisation[69]. This may indicate that the differential activation of the NF-κB pathway in macrophages can influence synovial pathotype and may affect lymphocyte interaction, although this needs to be investigated directly.

The pauci-immune pathotype (with no immune cell and only fibroblast infiltration) has been associated with poorer outcomes with anti-TNF therapy [56], but is the least well understood pathotype in terms of effectors of pathogenesis and drug targets. Fibroblast-like synoviocytes (FLS) have been shown to produce several pro-inflammatory cytokines including IL-6, IFN-γ and TNFα, usually in close interaction with lymphocytes and dendritic cells [70]. NF-κB partially controls FLS production of IL-6 [71, 72], and blockade of NF-κB activation by proteasome inhibition or IκBα transfection increases TNFα induced FLS apoptosis [73].

These studies strongly indicate that NF-κB drives synovial inflammation in all different subtypes/pathotypes of RA. Thus, NF-κB dysregulation and associated cell- and pathway-specific effects may be key contributors to the heterogeneous picture of pathotypes and disease spectrum.

4. **Role of NF-κB in the heterogeneity of Systemic Lupus Erythematosus**

Systemic Lupus Erythematosus (SLE) is a chronic, potentially life-threatening, inflammatory condition with multi-organ involvement and a spectrum of clinical phenotypes [74]. It is often characterised by autoantibody positivity – including anti-nuclear (ANA), anti-double stranded DNA (dsDNA), and anti-'Smith' antibodies. Plasmacytoid dendritic cells (pDCs) are the main producers of type 1 Interferon, which is induced by Toll like Receptor 7 (TLR) activation. Type 1 Interferon is a key factor in SLE pathogenesis, which has been shown to precipitate autoreactive B cell differentiation and secretion of

anti-nuclear antibodies [75]. Approximately half of patients with SLE express high interferon gene signatures [76], which led to the development of the recently FDA-approved monoclonal antibody anifrolumab [77] – an IFNAR1 antagonist. However, the phase III clinical trial demonstrated that efficacy of adjuvant anifrolumab was approximately 50% regardless of the interferon gene signature compared to 30% efficacy of standard care [77], suggesting that there are other pathogenic mechanisms. Aberrant TLR7 activation can be triggered by autoantigens leading in turn to activation of the transcription factor Interferon Regulatory Factor 3 (IRF3) and production of type 1 interferon, with IKKα implicated in the upstream phosphorylation to activate IRF3, independent of TLR7 induced NF-κB activation [78, 79]. This may be relevant to those patients who do not respond to anti-interferon-alpha therapy.

NF-κB has also been shown to be upregulated in autoreactive SLE B cells mediated via CD40 [80] and BAFF. BAFF is targeted by the monoclonal antibody, Belimumab, which ameliorates the severity of SLE nephritis [81]. As discussed in Section 2, several genetic variants in components of the NF-κB pathway have been associated with SLE. For example, B cells from SLE patients with the *UBE2L3* rs140490 were more sensitive to CD40 activation of NF-κB and clinically had an expansion of their plasma cell population [32]. The functional effects of the SLE genetic variants are largely still unknown in humans, but transgenic animal models give an insight into the possible effect of dysregulating the NF-κB pathway. B cell-specific deficiency of TRAF3, an upstream mediator of NF-κB, produces a lupus-like illness in mice with elevated serum double-stranded DNA antibodies, spontaneous germinal centre formation in the spleen, and increased lymphocytic infiltration of the kidney and liver [82].

TNF-related weak inducer of apoptosis (TWEAK) can also activate NF-κB and has been found to be elevated in the urine of lupus nephritis patients. Urinary TWEAK levels has been proposed as a surrogate marker of disease activity, as it correlates with increased renal expression of active phosphorylated p65 [83]. As patients without renal involvement do not require renal biopsy, the NF-κB activity in non-affected kidneys could not be investigated to confirm that the increased activity correlates only with the presence of nephritis. However, NIK inhibition in a lupus nephritis mouse

model did result in reduced proteinuria and a less severe glomerulonephritis on biopsy [84], which would support a key role for NF-κB in renal involvement.

5. **Role of NF-κB in the heterogeneity of Sjogren's Syndrome**

Sjogren's syndrome is an autoimmune condition affecting the exocrine glands and commonly presenting with symptoms of dry eyes and dry mouth [85]. Like SLE, it is strongly associated with lymphocyte proliferation – indeed, lymphoma is a serious potential complication of Sjogren's syndrome [86]. 'Activated' phosphorylated NF-κB components are elevated in salivary gland and PBMCs in Sjogren's patients but not healthy donors [87], whilst Sjogren's specific anti-Ro antibodies can induce canonical NF-κB activation in salivary gland epithelial cells [88]. Ectopic lymphoid structures can also form in the salivary gland in Sjogren's in a subset of patients [10]. Ping et al suggested that NF-κB is upregulated in epithelial cells surrounding the lymphocytic infiltration and that CD40 induces epithelial cell apoptosis partly through NF-κB activation [89].

NF-κB activity in epithelial cells appears to be instrumental in the development of Sjogren's. An epithelial cell-specific knockout of *Tnfaip3* (A20) in mice resulted in sialadenitis, with lymphocytic salivary gland infiltration and reduced saliva production [90], thought to be mediated by ectodysplasin mediated activation of NF-κB [91]. Different *TNFAIP3* polymorphisms have been associated with almost all SARDs. Although its exact role in the aetiology of this diverse range of condition remains unclear, this is unlikely a coincidence – future research must explore whether these unique polymorphisms may affect A20 function in a cell specific manner to localise disease.

6. **Role of NF-κB in the heterogeneity of other Systemic Autoimmune Rheumatic diseases**

Strikingly, NF-κB is upregulated in almost all SARDs, however the cells affected seem to have significant influence over the clinical phenotype of each condition. In a mouse model of small vessel crescenteric glomerulonephritis elevated nuclear NF-κB expression co-localised with endothelial cells in affected renal tissue. This vascular endothelial-specific role of NF-κB is reinforced with *in vitro*

human studies that demonstrate that neutrophils exposed to ANCA do not upregulate NF-κB, whilst endothelial cells exposed to ANCA-primed neutrophils do [92]. In systemic sclerosis, transcriptomic based subgroups have been proposed, in order to try and develop new therapeutic targets based on the transcription data, with fibroblast-related upregulation of NF-κB signalling pathways associated with the 'inflammatory' subset [93].

7. <u>**Relevance of NF-κB for existing treatments and potential roles in prediction of treatment response**</u>

Several existing conventional and biological DMARD therapies inhibit NF-κB activation to some degree. However, it is unclear to what degree this mechanism contributes to their efficacy, as all have other diverse effects on pro-inflammatory pathways. Anti-TNF and anti-IL1 receptor monoclonal antibody therapies directly block the activation of NF-κB by suppressing upstream activation [94]. Other common conventional DMARDs also suppress NF-κB: glucocorticoids can reduce nuclear NF-κB DNA binding [95]; sulfasalazine [96] and methotrexate [97] block phosphorylation of IκBα (resulting in NF-κB remaining bound in the cytoplasm); and leflunomide suppresses signalling downstream of NIK [98].

Given the wealth of literature reviewed above describing the influence of NF-κB in almost all SARDs, it seems an appealing biomarker to help stratify patient subgroups. A functional NF-κB activity assay on peripheral blood based on flow cytometry analysis of phosphorylated p65 has recently been postulated as a possible biomarker for anti-TNF non-response in cutaneous psoriasis. High pre-treatment LPS-induced NF-κB activity in conventional dendritic cells type 2 *ex vivo* correlated significantly with a poor response to anti-TNF therapy [99]. Although the mechanism for this is incompletely understood, NF-κB is a downstream effector of TNF and etanercept has been shown to reduce 'active' phosphorylated NF-κB in psoriatic plaques and lead to reduced plaque thickness [100]. It may be that in patients where NF-κB is activated in a TNF independent manner, they do not respond to anti-TNF therapy.

8. Novel therapeutic targets of NF-κB and related pathways in SARD

Despite many groups attempting to develop therapeutic small molecule NF-κB pathway inhibitors for both autoimmune disease and malignancy, very few have advanced to the clinical phase and none have been approved in clinical practice [101]. Targets have included inhibition of the IKK complex, NEMO, IRAK4, NF-κB nuclear translocation and post-translational modifications. Selective IRAK4 inhibitors have advanced to clinical trials with BAY 1830839 and PF-06650833, completing a Phase I and Phase II clinical trial, respectively, in Rheumatoid arthritis [102, 103], but results are still pending. IKKβ inhibitor, MLN-0415 was discontinued in Phase I trial due to incidence of adverse events [103]. However, the lack of progression of many of these small molecule NF-κB pathway inhibitors is associated with toxicity and severe side effect profile, most likely from broad blockade of a ubiquitous and key inflammatory transcription factor.

Bortezomib, a proteasomal inhibitor currently licenced for multiple myelomawas undergoing a phase IIa trial for refractory SLE and rheumatoid arthritis, but was terminated in 2019 due to recruitment difficulties [104]. However, blanket proteasomal inhibition and loss of the immunoregulation by the ubiquitin system can result in toxic side effects, including myelosuppression, cardiovascular toxicity and peripheral neuropathy. KZR-616, a dual immunoproteasome β5i/β2i proteasome inhibitor, is also undergoing phase Ib/II trial in SLE [105] and phase II trial in myositis (Trial ID: NCT04022926). This is expected to have a less toxic side effect profile due to more selective proteasomal inhibition. These therapies may still offer a desirable additional treatment option for life threatening refractory autoimmune disease (if successful at phase III trial), as a last-line treatment where the risk of the disease may outweigh the side effect profile.

Dimethylfumarate (DMF) has recently been shown to suppress linear ubiquitination with a higher degree of selectivity: it inhibits UBE2L3 with the highest affinity to block NF-κB activity, as well as upregulating the anti-oxidant transcription factor nuclear factor erythroid 2-related factor 2 (NRF-2) [106]. It is a key therapy for multiple sclerosis and licenced in refractory psoriasis treatment [107], and

has also been used off-label with some success in individual cases of refractory cutaneous lupus erythematosus [108]. Therefore, inhibiting the ubiquitination process may present a more promising potential novel therapeutic target in the NF-κB pathway.

9. Concluding remarks

Dysregulated NF-κB has long been implicated in the pathogenesis of autoimmune rheumatological disease. However, it is increasingly clear that differential activation of the canonical and non-canonical pathways in a cell-specific manner can result in drastically different molecular patterns and organ involvement, which can in turn influence the clinical phenotype. This cell-specific approach has been adopted in the recently reported functional NF-κB activity assay in conventional type 2 dendritic cells, where high pre-treatment NF-κB activity was associated with a poor anti-TNF outcome in cutaneous psoriasis.

Current conventional and biologic therapies target a variety of pro-inflammatory pathways and are not specific to NF-κB alone. Such non-selective mechanisms are associated with significant side-effects. Given the constitutive role of NF-κB in maintaining immune homeostasis and the physiological response to inflammation, targeted therapies involving NF-κB inhibition present a promising avenue for future advancement. However, before this potential can be realised there is an urgent need for further research to fully characterise associations between NF-κB dysregulation and individual rheumatic diseases, and furthermore identify whether heterogeneity in patterns of NF-κB dysregulation across pathogenic tissue-specific cell types underlies the development of different diseases and potentially divergent subgroups or endotypes within each disease.

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