Non-canonical NF-κB signaling in rheumatoid arthritis and beyond

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Non-canonical NF-κB signaling in rheumatoid arthritis: Dr. Jekyll and Mr. Hyde?

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ABSTRACT
The nuclear factor-kappaB (NF-κB) family of transcription factors is essential for the expression of pro-inflammatory cytokines, but can also induce regulatory pathways. NF-κB can be activated via two distinct pathways: the classical or canonical pathway, and the alternative or non-canonical pathway. It is well established that the canonical NF-κB pathway is essential both in acute inflammatory responses and in chronic inflammatory diseases, including rheumatoid arthritis (RA). Although less extensively studied, the non-canonical NF-κB pathway is not only central in lymphoid organ development and adaptive immune responses, but is also thought to play an important role in the pathogenesis of RA. Importantly, this pathway appears to have cell type-specific functions and since many different cell types are involved in the pathogenesis of RA, it is difficult to predict the net overall contribution of the non-canonical NF-κB pathway to synovial inflammation. In this review, we describe the current understanding of non-canonical NF-κB signaling in various important cell types in the context of RA and consider the relevance to the pathogenesis of the disease. In addition, we discuss current drugs targeting this pathway, as well as future therapeutic prospects.

Keywords
NF-κB, non-canonical, rheumatoid arthritis, synovial tissue, treatment
INTRODUCTION

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a disabling chronic inflammatory autoimmune disease, affecting the synovial joints. Most patients have considerably raised levels of serum immunoglobulins and have characteristic auto-antibodies against autologous immunoglobulin, called IgM-rheumatoid factor (RF), and anti-citrullinated protein antibodies (ACPA). In the early phase of the disease, the synovial tissue is infiltrated by immune cells and increases in thickness which causes pain, stiffness and swelling of the joint. The synovial cell infiltrate contains various lymphocytes, plasma cells, macrophages, and other cells \(^1\). T-helper lymphocyte populations in the joints are oligoclonal and have been demonstrated to be autoreactive \(^2,3\). The immune cells produce cytokines that also activate stromal cells such as fibroblast-like synoviocytes and endothelial cells. These cells contribute to the inflammatory process via the production of matrix metalloproteinases (MMPs), cytokines and chemokines, followed by the influx and activation of more immune cells into the synovial tissue \(^4\). From the earliest stage of the disease, neoangiogenesis can be observed which contributes to chronicity. In chronically inflamed joints, osteoclasts are activated that contribute to erosion of bone. The debris resulting from bone and cartilage degradation may be a further source of synovial inflammation, leading to a vicious cycle. Eventually, the loss of articular cartilage, along with damage to the joint capsule and peri-articular structures, causes deformities (reviewed in \(^5\)).

In RA synovial tissue many signal transduction pathways are activated \(^6\). One of the most important signaling pathways involved in the pathogenesis of RA is the nuclear factor-κB (NF-κB) pathway (reviewed in \(^4,6,7\)).

Nuclear Factor-κB

NF-κB was first discovered in 1986 in the nucleus of B cells as an enhancer of the κB immunoglobulin chain \(^8\). It has since been shown to be expressed ubiquitously in the cytoplasm of almost all cell types \(^8,9,10\) and NF-κB appeared to be a major regulator of innate and adaptive immunity and inflammatory responses \(^11\). Many diseases, including cancer, inflammatory and autoimmune diseases, are associated with dysregulation of NF-κB (reviewed in \(^12\)). NF-κB can be activated via two distinct pathways, the classical or canonical NF-κB pathway, and the alternative or non-canonical NF-κB pathway.

The canonical NF-κB pathway

The most extensively studied NF-κB activation pathway is the canonical pathway (Figure 1), which can be activated by stimulation of a variety of cell membrane receptors, including tumor necrosis factor (TNF) receptor, IL-1 receptor, and Toll-like receptors, in response to pro-inflammatory stimuli like LPS, IL-1 and TNF, as well as via triggering of the T-cell receptor or B-cell receptor. In this pathway, inhibitor of κB (IκB) kinase (IKK) \(\beta\) is required for NF-κB activation, whereas IKKa is redundant for the activation and
induction of NF-κB DNA-binding activity. The canonical NF-κB pathway is essential both in acute inflammatory responses and in chronic inflammatory diseases such as RA and inflammatory bowel disease. Activation of this pathway results in transcription of genes encoding important components of the innate immune response, such as chemokines, cytokines, MMPs, adhesion molecules and inhibitors of apoptosis. The importance of this pathway is supported by a number of studies that showed a significant increase in susceptibility to bacterial infections in RelA- and IKKβ-deficient mice. The canonical NF-κB pathway is also essential for the expression of pro-inflammatory cytokines in many immune cells, including dendritic cells (DC). Moreover, this pathway is important in cell proliferation and survival, demonstrated by constitutively active NF-κB signaling in many tumor tissues. In RA IKKβ is a key regulator of synovial inflammation. In line with this, the canonical pathway plays an important role in the development and perpetuation of inflammation in RA. Canonical NF-κB subunits RelA/p50, are observed in RA synovial tissue. In RA, inflammation is driven by NF-κB dependent inflammatory cytokines like TNF, IL-1 and IL-6 that are produced by synovial fibroblasts and macrophages. DC in RA synovial tissue have an activated phenotype, associated with expression of costimulatory molecules and production of pro-inflammatory cytokines. In addition, leukocyte migration into the inflamed tissues is controlled through activation of the classical NF-κB pathway and upregulation of adhesion molecules, such as ICAM-1.

Consequently, targeting this pathway in (pre)clinical models of arthritis has been proven very beneficial. The importance of the canonical NF-κB pathway in RA is underlined by the common and successful use of anti-TNF therapy, one of the main target genes of the canonical NF-κB pathway. The canonical NF-κB pathway not only plays an important role in the initiation and progression of RA, but also in other inflammatory diseases, such as inflammatory bowel disease and asthma, and cancer. However, this is outside the scope of the current review, but discussed in more detail in previous reviews. Here, we will focus on the alternative or non-canonical NF-κB pathway.

The non-canonical NF-κB pathway
In the last decade, a second, alternative NF-κB activation pathway has been identified, the so-called non-canonical NF-κB pathway (Figure 1). This pathway can be triggered by the activation of members of the TNF-receptor superfamily including the lymphotoxin β receptor (LTβ-R), CD40, B cell activating factor belonging to the TNF family (BAFF) receptor, and receptor activator of NF-κB (RANK). Of note, ligands of the TNF-receptor superfamily simultaneously also activate the canonical pathway and regulated cross-talk between both pathways exists at different levels. The non-canonical NF-κB pathway is strictly dependent on IKKa homodimers and unlike the canonical pathway does not involve IKKβ or IKKγ. In the steady state, NF-κB inducing kinase (NIK), the most important kinase of the non-canonical pathway, is continuously degraded. TRAF3 mediates recruitment of NIK to TRAF2, which recruits the E3 ligases cIAP1 and cIAP2, and ubiquitination of NIK by these cIAPs then promotes its proteosomal degradation. Consequently, endogenous...
levels of NIK are very low\textsuperscript{37,38} and the NF-κB complex is retained in the cytoplasm and kept inactive. Recently, a second level of regulation has been identified via the deubiquitinase OTUD7B. Upon activation of the non-canonical NF-κB pathway, OTUD7B binds and deubiquitinates TRAF3, thereby inhibiting TRAF3 proteolysis and preventing aberrant non-canonical NF-κB activation\textsuperscript{39}. The importance of tightly regulated NIK is illustrated by the lethality of TRAF3-deficient mice\textsuperscript{35}.

Upon signal-induced activation of the non-canonical NF-κB pathway, TRAF2 ubiquitinates and activates cIAP1-cIAP2 to induce proteolysis of TRAF3. Degradation of TRAF3 prevents targeting of newly synthesized NIK, resulting in accumulation of NIK. Subsequently, NIK induces processing of p100 by IKKα homodimers, resulting in partial degradation into p52. Next, mainly p52-RelB heterodimers translocate to the nucleus, leading to target gene transcription (reviewed in\textsuperscript{12,33}). Whereas canonical NF-κB activation is rapid and independent of protein synthesis, non-canonical NF-κB activation requires NIK synthesis and accumulation. Consequently, the kinetics of this pathway are considerably slower (reviewed in\textsuperscript{12,33}).
Of note, ligands of TNF-receptor superfamily member simultaneously also activate the canonical pathway and regulated cross-talk between both pathways exists at different levels. IKKα has for instance been described to also have nuclear functions and serves as a regulator of canonical NF-κB dependent gene expression through control of promoter-associated histone phosphorylation after cytokine exposure. However, in macrophages IKKα also terminates canonical NF-κB dependent transcription of target genes by accelerating both the turnover of the NF-κB subunits RelA and c-Rel, and their removal from pro-inflammatory gene promoters. Reciprocally, canonical NF-κB activity suppresses basal non-canonical NF-κB signaling in immune cells. Interestingly, it has been shown that under certain circumstances also other stimuli, including TNF, can to some extent activate non-canonical NF-κB signaling in specific cell types, and IKKα is critical for interferon-α production induced by Toll-like receptors.

The functional role of the non-canonical NF-κB pathway in vivo has been established by the identification of phenotypic abnormalities shared by knockout mice or mutants of specific signaling molecules involved in this pathway. TRAF2-deficient mice die prematurely and show elevated serum levels of TNF. Loss of TRAF3 results in constitutive processing of p100, leading to constitutive non-canonical NF-κB activation. This results in early postnatal lethality in TRAF3-deficient mice. Nik⁻/⁻ mice and aly/aly mice that are homozygous for a spontaneous recessive point mutation in the gene encoding NIK, both lack lymph nodes. Furthermore, these mice have reduced humoral and cell-mediated immune functions, and are more susceptible to infections, although they do have mature T and B cells. IKKα⁻/⁻ mice are lethal, or die within 30 minutes after birth. Loss of IKKα results in severe defects in limb, skeletal and Peyers’ patches development in the embryo. In p100⁻/⁻ mice that lack p100 but still containing a functional p52 protein, the non-canonical NF-κB pathway is constitutively activated. These mice have enlarged lymph nodes, abnormal lymphocyte proliferation, and suffer from gastric hyperplasia resulting in early postnatal death. Furthermore, these mice show severe defects in B-cell function, and impairment in the formation of proper architecture in peripheral lymphoid organs. RelB⁻/⁻ mice develop normally until 4 - 10 weeks after birth, but then skin lesions and hair loss start to occur. These mice exhibit a complex inflammatory phenotype, myeloid hyperplasia, and splenomegaly. These studies demonstrate that the non-canonical NF-κB pathway controls the expression of genes involved in B-cell function and lymphoid organogenesis, as well as cell proliferation and survival. Recently, a role for the non-canonical NF-κB pathway in the regulation of immune responses has been proposed. IKKa is implicated in the negative regulation of inflammatory responses in macrophages and DC. NIK has a role in the development of regulatory T cells (Tregs) and contributes to the expression of the immunoregulatory enzyme indoleamine-2,3-dioxygenase in DC.

Overall, this pathway is crucially involved in lymphoid organ development and adaptive immune responses. The role of non-canonical NF-κB signaling in (synovial) inflammation is addressed below.
Non-canonical NF-κB signaling in rheumatoid arthritis

In the inflamed RA synovial tissue, all stimuli that are able to induce non-canonical NF-κB signaling are abundantly present. Lymphotoxin β (LTβ) plays an essential role in lymphoid organ development and it has been demonstrated that this is dependent on LTβR induced non-canonical NF-κB activation (reviewed in 58). In RA synovial tissue, tertiary lymphoid structures (TLS) can be formed that often resemble germinal centers 59, in which B and T cells cluster in aggregates with surrounding fields of plasma cells. In approximately 20-30% of RA patients even larger aggregates of immune cells containing FDC can be found. In line with this, LTβ is also important in the formation of TLS in RA synovitis 34,60,59. Signaling through the LTβR can also be induced by LIGHT and high levels of LIGHT have been observed in serum 61, synovial tissue 62, as well as in synovial fluid of RA patients 62,61. In addition, LIGHT is upregulated on B cells and monocytes in RA peripheral blood and synovial fluid 63.

Activation of the non-canonical NF-κB pathway can also be induced via BAFF/BAFF-R or CD40L/CD40 triggering. CD40L is highly expressed in RA synovial tissue 64, and elevated in serum and peripheral blood of RA patients 65. CD40L forms trimers and therefore promotes trimerization of the receptor 66. Furthermore, CD40L and CD40 are highly expressed by T lymphocytes and macrophages in the synovial fluid of RA patients 67. In patients with RA, SLE and Sjogren's syndrome, elevated serum levels of BAFF have been observed (reviewed in 68,69). Also, BAFF/BAFF-R are widely expressed in RA synovium 70 and BAFF is present in synovial fluid of RA patients 71.

Interaction of RANK and RANK ligand (RANKL) has been extensively studied in RA because of the important role in osteoclast biology. RANKL is highly expressed in synovial tissue of RA patients with active disease 72 not only in the synovial sublining and within areas of lymphocyte infiltration 72, but also at the pannus-bone interface at sites of articular bone erosion 73. In addition, increased levels of soluble RANKL are found both in serum 74 and synovial fluid from RA patients 75,76.

Together, these studies suggest that activators of the non-canonical NF-κB pathway play an important role in RA synovial inflammation, although direct involvement of the non-canonical NF-κB pathway is hitherto lacking. Nik−/− mice and aly/aly mice that are homozygous for a spontaneous recessive point mutation in the gene encoding NIK, both lack lymph nodes and have a disturbed microarchitecture of the spleen. Furthermore, these mice have reduced humoral and cell-mediated immune functions, and are more susceptible to infections, although they do have mature T and B cells 48,49. Relb−/− mice develop a complex phenotype, including an autoimmune-like inflammatory syndrome, myeloid hyperplasia, multifocal defects in immune responses, and impaired development of lymphoid organs 56,77,78. These studies demonstrate that the non-canonical NF-κB pathway controls the expression of genes involved in B-cell function and lymphoid organogenesis, as well as cell proliferation and survival. NIK has also been shown to be critical for inflammation-induced osteoclastogenesis, as NIK-deficient mice were largely resistant to arthritis with less osteoclastogenesis and less bone erosion 79. Importantly, the non-canonical NF-κB
pathway appears to play different roles in different cell types. Since many cell types are involved in the pathogenesis of RA, it is very difficult to predict the net overall contribution of non-canonical NF-κB signaling to synovial inflammation. Therefore, the role of the non-canonical NF-κB pathway in the most important cell types involved in RA synovial inflammation is discussed below.

Macrophages
In the inflamed RA synovial tissue a large number of macrophages is present that contributes to inflammation, which is substantiated by our observation that the number of CD68+ synovial sublining macrophages correlates with clinical improvement independently of the therapeutic strategy. However, macrophages are phenotypically heterogeneous, which is dictated by the microenvironment. They are not only the main producers of proinflammatory cytokines like TNF, IL-1, IL-15, IL-18, IL-23, and IL-27 that play an important role in the persistence of inflammation, but also generate inhibitory mediators such as IL-10, transforming growth factor-β, and soluble TNF receptor (reviewed in ). We and others have demonstrated that the canonical NF-κB pathway plays an important role in macrophage production of TNF-α and destructive matrix metalloproteinases, whereas the production of the anti-inflammatory cytokines IL-10 and IL-1RA is independent of canonical NF-κB signaling.

Several groups have studied the role of the non-canonical NF-κB pathway in macrophages. The non-canonical NF-κB pathway is activated during human monocyte-macrophage differentiation and the increase in IKKα may act as a brake preventing hyperactivation of the new macrophage. IKKα accelerates turnover of classical NF-κB subunits RelA and c-Rel in macrophages, and their removal from pro-inflammatory gene promoters, thereby negatively regulating its own activation. Consequently, inactivation of IKKα in mice resulted in enhanced inflammation and bacterial clearance. These data strongly suggest that the non-canonical NF-κB pathway plays a regulatory role in macrophages. In contrast to these findings, NIK has also been shown to induce phosphorylation of IKKα and subsequently induce phosphorylation of histone H3. In a previous study these authors showed that histone modification in macrophages is important for COX-2 expression, which is responsible for the production of prostaglandins that are involved in inflammation. These data imply that the non-canonical NF-κB pathway plays an important role in the modification of nucleosomal structure and via this mechanism contributes positively to inflammation. In addition, a similar role for NIK was proposed in regulating COX-2 gene expression in macrophages. Binding of NIK to the transcription factor PU.1, which is located in the nucleus and is activated by phosphorylation to bind to cognate DNA elements, resulted in increased binding of activated PU.1 to the COX-2 promoter in response to treatment with endotoxin. Together, these findings suggest a role for NIK in mediating pro-inflammatory COX-2 gene expression in macrophages. Another, indirect pro-inflammatory role for the non-canonical NF-κB pathway was established in mouse bone marrow-derived macrophages. Here, the non-canonical NF-κB pathway was
critical for production of the chemokine CXCL12 that is required for cells to migrate toward HMGB1, a proinflammatory cytokine and chemoattractant for immune effector cells.

In RA synovial tissue, the abundant presence of non-canonical NF-κB ligands is likely to induce signaling in macrophages as well. LIGHT is expressed in CD68+ macrophages and in vitro stimulation of these cells with LIGHT induced expression of MMP-9 and pro-inflammatory cytokines TNF-α, IL-6, and IL-8. Synovial fluid macrophages express increased levels of CD40 and produce both IL-12p40, TNF-α and IL-10 after CD40 ligation. However, the contribution of the non-canonical NF-κB pathway to these LIGHT- and CD40L-induced processes remains to be tested. In contrast to other leukocytes, macrophages are more long-lived cells and also persist at inflammatory lesions during the resolution of inflammation. Therefore, the non-canonical NF-κB pathway may play a dual role in macrophages and can act both as a pro-inflammatory and as an anti-inflammatory pathway. Consequently, when targeting this pathway in macrophages it is hard to predict what the net effect would be in RA.

Dendritic cells

In RA synovial tissue, immature and mature dendritic cell (DC) subsets can be observed in close association with T cells and B cell follicles. These DC may contribute to ongoing inflammation through presentation of autoantigens or production of pro-inflammatory cytokines. ACPA and IgM-RF are presumed to bind to Fc receptors on macrophages and DC, inducing their activation and production of pro-inflammatory cytokines. NF-κB is essential for normal DC differentiation, activation and survival. CD40-ligation on DC induces early production of inflammatory cytokines via the canonical NF-κB pathway, as well as late expression of the anti-inflammatory enzyme indoleamine 2,3-dioxygenase (IDO) via non-canonical NF-κB signaling, which is able to suppress T cell activation and to promote T cells with regulatory functions. Interestingly, both synovial fluid and synovial tissues of RA patients contain DC that express functional IDO, pointing towards a possible mechanism of tolerance in RA synovial inflammation. In addition, Grohmann et al. demonstrated that plasmacytoid DC possess GITRL and that reverse signaling through GITRL results in non-canonical NF-κB activation and IDO-dependent protection in an allergic airway inflammation mouse model. These results are consistent with in vivo experiments performed in mice lacking functional NIK that have a decreased number of DC which also have lower expression of costimulatory molecules and exhibit reduced antigen presentation. Consequently, these DC have a decreased ability to induce expansion of CD25+CD4+ regulatory T cells. This would suggest that NIK expression in DC is important to maintain CD25+CD4+ regulatory T cell numbers. At the same time, it was demonstrated that DC require non-canonical NF-κB signaling to cross-prime CD8+ T cells to exogenous antigen, whereas MHC class II presentation and migration occurs independently of NIK. These experiments point to a role for NIK in DC in cross-priming of soluble antigen. Furthermore, in mice NIK expression in DC is also important for supplying co-stimulatory signals to CD4+ T cells and non-canonical
NF-κB signaling is required in DC to generate effector T cells. However, in human DC NIK was not essential for effective antigen presentation. Interestingly, NF-κB2/p100 has a crucial role in the negative regulation of RelB-induced DC maturation and may function to prevent DC hyperactivation, which indicates that the non-canonical pathway can also negatively regulate CD4+ T cell mediated adaptive immune responses.

Taken together, the non-canonical NF-κB pathway regulates both pro-inflammatory and anti-inflammatory processes in DC. The net result is likely to rely heavily on additional stimuli and the microenvironment in which the cells are present.

**B cells and plasma cells**

B cells are present in the inflamed RA synovial tissue in close contact with T cells. The efficacy of anti-CD20 treatment has confirmed the pivotal role of B cells in the pathogenesis of RA, both in mice and in patients. The canonical NF-κB pathway was originally described in B cells and is crucial for B cell development, maintenance, and function (reviewed in). However, the non-canonical pathway also plays an important role in B cell biology. TRAF3−/− B cells show increased expression of ICAM-1 and protection from spontaneous apoptosis during in vitro culture. IKKa in B cells is crucial for germinal center formation, and long-lived immunoglobin titers, but not for primary antibody production. However, aly/aly mice that display a functional defect in NIK do have reduced serum Ig levels and do not show class switching to IgA, which may in part be due to failure of lymphocytes to migrate to the proper microenvironment where B cells proliferate and differentiate into Ig-producing cells. Studies in NIK-deficient mice showed that B cells were defective in inducing class switching to IgA after anti-CD40 and BAFF stimulation, as measured by the frequency of IgA+ B cells, but were capable of TGF-β-induced class switching to IgA. Additional studies in Nfkb2<sup>−/−</sup>Lym1/Lym1<sup>−/−</sup> mice, a mutant mouse strain that due to a Lym1 mutation in the gene encoding p52 produce a nonprocessable form of p100 resulting in a blockade of non-canonical NF-κB activation, confirmed these findings. These results further underline that the non-canonical NF-κB pathway has an important role in regulating antibody production and class switching to IgA. Importantly, NIK also regulates BAFF-mediated expression of inducible costimulator ligand (ICOSL), a molecule required for follicular helper T cell generation. In addition, NIK provides survival signals in B cells, which is illustrated by the fact that B cell-specific ablation of TRAF2 and TRAF3 (negative regulators of NIK) markedly enhanced the survival and proliferation of B cells. Furthermore, non-canonical NF-κB signaling also regulates plasma cell generation, proliferation and survival. Under normal, physiological conditions, signal transduction events activated through CD40 and BAFF-R are necessary for the induction of p100 proteolysis and for the promotion of B-cell survival. Consistent with this notion, B-cell specific ablation of TRAF2 and TRAF3 (negative regulators of NIK) markedly enhances the survival and proliferation of B-cells. This was confirmed by another study that also demonstrated that inhibition of both cIAP1 and cIAP2 was required for the non-canonical NF-κB mediated increased survival and proliferation of primary B lymphocytes. In addition, non-canonical NF-κB
signaling also regulates plasma cell generation, proliferation and survival. In B cells, LIGHT stimulation results in enhanced CD40L-induced proliferation and enhances Ig secretion. However, it was not formally tested whether these effects were dependent on LTβR triggering and subsequent (non-)canonical NF-κB signaling or HVEM signaling.

In conclusion, the non-canonical pathway plays an important role in B cells and plasma cells by promoting survival, differentiation and antibody production, which is likely to contribute to the persistence of inflammation in RA.

**T cells**

In RA synovial tissue many T cell subsets can be found, including Th1, Th17 and Tregs (reviewed in). They express an array of molecules on their surface that are involved in effector function (IL1R, TNF, RANKL), costimulation (CD28), antigen recognition (TCR), memory effector function and several chemokines and chemokine receptors. The canonical NF-κB pathway is well-known for its role in the activation and differentiation of T cells. In contrast, the role of the non-canonical NF-κB pathway in T cell function is less well studied. It has been shown that NIK and RelB are essential for T cell activation via the TCR/CD3 pathway. NIK and IKKa have a T-cell intrinsic function in the regulation of Th17 cells, a subset of CD4+ effector T cells that produce IL-17 family of cytokines, which are intimately involved in autoimmunity, including RA. Nik-/- mice are resistant to experimental autoimmune encephalomyelitis (EAE), a disease model that involves Th17 cells. This function of NIK in mediating EAE was demonstrated to be T-cell intrinsic and implicates NIK as a critical signaling molecule that regulates Th17 differentiation and EAE induction. Another study also found that NIK is essential for T cell function in vivo and that its tight regulation in T cells is crucial to prevent autoimmunity in mice. The non-canonical NF-κB pathway is also required for the generation of CD4+ T-follicular helper cells mediating B-cell activation and antibody responses. In contrast, NIK is essential for the generation of regulatory T cells that dampen inflammatory responses as well. However, NIK is not required for the suppressive function of these CD4+CD25+ T cells. In another study it has been shown that regulatory T cells from NIK-deficient mice display hyperproliferative activities upon GITR stimulation, while their suppressive capacities remain intact. These data suggest a novel role of NIK in controlling the development and expansion of CD4+CD25+ regulatory T cells.

In summary, in T cells a dual role for the non-canonical NF-κB pathway exists, which is not only important in T cell activation and the induction of Th17 cells, but is also required for the generation of regulatory T cells.

**Osteoclasts**

Maintenance of healthy bone requires the balanced activities of osteoclasts that resorb bone and osteoblasts that build bone. In RA synovial tissue, osteoclasts that resorb bone are found at sites adjacent to bone, causing local bone destruction. Interestingly, aly/aly mice that lack functional NIK have an increased bone mineral density and bone...
volume. In addition, these mice and Nik−/− mice have a significant defect in RANKL-induced osteoclastogenesis in vitro and in vivo. Similar effects were observed in osteoclasts derived from bona fide Nik−/− mice. Also, Nik−/− mice exhibit significantly less periarticular osteoclastogenesis and less bone erosion in the serum transfer arthritis model. In antigen-induced arthritis, development of disease was completely impaired. Interestingly, overexpression of constitutively active IKKα or p52 restored osteoclastogenesis in aly/aly cells and re-introduction of RelB, but not p65 in relB−/− cells rescued osteoclast formation. Interestingly, deletion of RelB in mice results in significantly increased bone mass due to decreased osteoclast activity and increased osteoblast numbers, indicating that RelB may also have a role in bone formation. Activated osteoclasts are responsible for the bone loss associated with RA. Constitutive activation of NIK drives enhanced osteoclastogenesis and bone resorption, both in basal conditions and in response to inflammatory stimuli. p100−/− mice that specifically lack the p100 inhibitor, but still express p52 resulting in increased non-canonical NF-κB signaling, also exhibit an osteopenic phenotype due to increased osteoclast and decreased osteoblast numbers. In humans, RANKL expressed by synovial fibroblasts or T cells, effectively induces osteoclast formation. Also, IKKα is required for RANKL-induced osteoclast formation in vitro. Interestingly, LIGHT not only promotes RANKL-dependent osteoclast formation, but also can independently induce a significant amount of osteoclast formation and bone resorption. These findings suggest that LIGHT may also be involved in the progression of inflammatory bone destruction in RA.

In conclusion, non-canonical NF-κB signaling in osteoclasts unambiguously contributes to bone destruction in RA, suggesting that this pathway may be an interesting target in these cells.

**Synovial fibroblasts**

Fibroblasts are connective tissue cells that show a remarkable plasticity to differentiate into other cell types like cartilage, adipocytes or smooth muscle cells. RA synovial fibroblasts (RASF) create an abnormal stromal microenvironment that is thought to be crucial for the persistence of inflammation, not only by architecture, but also by secreting pro-inflammatory cytokines or by producing growth factors that stimulate neovascularization (reviewed in). In addition, it has been demonstrated that RASF display certain unique features, such as an invasive, tumor-like behavior that is normally not observed in fibroblasts (reviewed in). Canonical NF-κB signaling contributes to IL-6, IL-8, ICAM-1, and MMP production by RA synovial fibroblasts and provides an important survival signal that suppresses apoptosis in these cells. Interestingly, non-canonical NF-κB signaling has been demonstrated to contribute to inflammation in RASF, since NIK is essential for LTβ-R activation of NF-κB in these cells. Also, stromal cells from mice lacking functional NIK showed decreased levels of adhesion molecules and increased CXCL13 expression. These findings suggest that NIK activity in stromal cells may play an important role in regulating the migration of immune cells. Stimulation of RASF with LIGHT results in
upregulation of adhesion molecules and MMPs. Also, CD40 ligation of RASF induces RANKL expression resulting in enhanced osteoclast formation. In RASF stimulation with CD40L also induces the non-canonical pathway target gene CXCL12 that may promote angiogenesis and the migration of T cells, B cells, and monocyte/macrophages into the inflamed synovium.

In summary, non-canonical NF-κB signaling in RASF contributes to the pathological behavior of these cells, leading to persistence of inflammation.

Endothelial cells

Endothelial cells (EC) play a crucial role in the pathogenesis of RA. First, they express adhesion molecules and produce chemokines thereby acting as the site of entry for immune cells such as lymphocytes, neutrophils and dendritic cells into the synovial tissue. In addition, EC proliferate and give rise to neovascularization that contributes to the persistence of inflammation (reviewed in 147). In synovial tissue containing TLS, also highly differentiated EC such as high endothelial venules (HEV) can be observed. Consequently, the signaling pathways that control activation of EC have been extensively studied and the canonical NF-κB pathway was demonstrated to play an important role in the expression of many of the pro-inflammatory genes. Madge et al. reported that LIGHT and LTα1β2, but not TNF, induce CXCL12 expression in EC, which required non-canonical NF-κB signaling. CXCL12 expression in EC has been demonstrated in RA synovial tissue as well. In addition, CXCL12 colocalized with αvβ3, a marker for neoangiogenesis, in RA EC, which would suggest that CXCL12 may be involved in RA synovial tissue angiogenesis. We have observed that CXCL12 is expressed by NIK+ blood vessels in RA synovial tissue. Subsequently, we established that non-canonical NF-κB signaling in EC stimulates pathological angiogenesis. Recently, it was demonstrated that EC-specific LTβR signaling is crucial for lymph node and HEV formation in mice, designating EC as an important player in organizing lymphoid tissue. Results from our group further support these data, as we observed high numbers of NIK+ EC in TLS in RA synovial tissue and correlated this with the presence of perivascular (pre-) follicular dendritic cells. This suggests that these cells may be important in the formation of TLSs in chronic inflammation.

In summary, non-canonical NF-κB signaling in EC is likely to contribute to angiogenesis and the influx of immune cells into the inflamed RA synovial tissue, thereby perpetuating the inflammatory response. Therefore, blocking this pathway in EC is probably beneficial in RA.

Mast cells

In RA the few mast cells in the normal synovium expand to constitute 5% or more of all synovial cells. Mast cells are mostly found around blood vessels and in the sublining area, but also in sites of cartilage erosions and in the synovial fluid. They are involved in the inflammatory process and mast cell-derived mediators can induce edema, destroy
connective tissue, and are involved in lymphocyte chemotaxis and in pathological fibrosis of RA joints. Moreover, mast cells are involved in angiogenesis, and their proteolytic activity results in cartilage destruction and bone remodeling. The production of some of these mediators such as IL-6, IL-8, TNF and MMPs is at least in part mediated by the canonical NF-κB pathway. In addition, IKKβ is involved in IgE-mediated degranulation of mast cells. The role of non-canonical NF-κB signaling in mast cells has not been studied extensively. Interestingly, LTβR triggering in mast cells by LIGHT produced by activated T cells induces pro-inflammatory cytokine and chemokine release. Therefore, stimuli that induce non-canonical NF-κB signaling in mast cells may augment inflammatory cascades by the enhanced secretion of cytokines and chemokines with paracrine and/or autocrine functions.

**Targeting the non-canonical NF-κB pathway in rheumatoid arthritis**

Despite the diverse roles of the non-canonical NF-κB pathway in different cell types, overall this pathway is likely to contribute to the persistence of inflammation in RA. As alluded to earlier, non-canonical NF-κB signaling can be induced via triggering of various TNF-receptor superfamily members, such as LTβR, CD40, BAFF-R, and RANK by their respective ligands. The important role of these ligand-receptor pairs in (synovial) inflammation is illustrated by the numerous attempts of pharmaceutical companies to target these pathways not only in RA, but also other disease like cancer. Below, we will discuss compounds that have been tested in RA patients or preclinical models of RA (table 1).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
<th>Biologic</th>
<th>Type of agent</th>
<th>Activity of biologic</th>
<th>Stage of development</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAFF</td>
<td>BAFF-receptor</td>
<td>Belimumab (Benlysta; Human Genome Sciences/GlaxoSmithKline)</td>
<td>Human BAFF-specific antibody</td>
<td>antagonist</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tabalumab/LY2127399</td>
<td>Human BAFF-specific antibody</td>
<td>antagonist</td>
<td>Phase II</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40</td>
<td>BI 655064</td>
<td>Humanized CD40-specific antibody</td>
<td>antagonist</td>
<td>Phase I in progress</td>
</tr>
<tr>
<td>LTα</td>
<td>LTβR</td>
<td>Baminercept</td>
<td>Human LTβR-IgG1 fusion protein</td>
<td>antagonist</td>
<td>Phase II</td>
</tr>
<tr>
<td>LTα</td>
<td>LTβR</td>
<td>Patecluzimab</td>
<td>Humanized LTα-specific antibody</td>
<td>Depleting and antagonist</td>
<td>Phase II in progress</td>
</tr>
<tr>
<td>RANKL</td>
<td>RANK</td>
<td>Denosumab (Prolia/Xgeva; Amgen)</td>
<td>Human RANKL-specific antibody</td>
<td>antagonist</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

BAFF, B cell activating factor; BAFFR, BAFF receptor; CD40L, CD40 ligand; Ig, immunoglobulin; LTα, lymphotoxin-α; LTβR, LTβ receptor; RANK, receptor activator of NF-κB; RANKL, RANK ligand.
**Anti-BAFF/BAFF-R treatment**
Belimumab, a fully human IgG1 monoclonal antibody to BAFF was tested in RA in a phase II placebo-controlled dose-ranging trial. Due to the modest clinical response in this trial, it was not further tested for RA. However, belimumab has been proven to have a beneficial clinical effect in systemic lupus erythematosus (SLE) patients, and is licensed for use in SLE in the United States, Canada, and Europe. A different anti-BAFF monoclonal antibody, tabalumab, has also been tested in patients with active RA. This drug showed modest efficacy, but nevertheless clinical development in RA was discontinued.

**Anti-CD40/CD40L treatment**
Clinical trials targeting the CD40/CD40L interaction in RA are currently ongoing, but so far have not been published. However, animal models have demonstrated encouraging results. In the CIA model, treatment with agonistic CD40 antibodies at the time of CIA induction exacerbates disease. In contrast, treatment with anti-CD40L antibodies prior to CIA induction ameliorated development of disease, resulting in less synovial inflammation, and less bone erosion. In addition, anti-CD40L treatment in the K/BxN arthritis model diminished arthritis development when administered a week before the onset of disease. Of note, no effect on disease activity was observed when animals were treated after clinical onset of disease. In humans, two clinical studies investigating the effects of anti-CD40L treatment have been conducted in patients with SLE. Both ruplizumab and toralizumab showed promising clinical and laboratory responses in some SLE patients. However, one of these studies was discontinued prematurely because of thromboembolic events. Since then, new reagents inhibiting CD40L-mediated events that are less likely to increase the risk of thromboembolic complications have been developed and clinical trials in RA patients are in progress.

**Anti-LTβ/LTβR treatment**
In the collagen-induced arthritis model, treatment with a LTβR-Ig fusion protein, severity of disease and joint tissue damage was reduced.

The efficacy and safety of baminercept, a LTβR-IgG fusion protein, was evaluated in patients with RA, in a placebo-controlled, phase IIb trial. However, baminercept did not exhibit measurable clinical effects in RA patients. Pateclizumab, a humanized mouse antibody, specifically binds LTα in both the soluble LTα3 homotrimeric form and the surface-expressed LTα1β2 heterotrimer, thereby interfering with binding of LT trimers to their cognate receptors, was tested for safety and efficacy in a phase I study. Preliminary beneficial effects were observed in RA patients and a phase II trial to further test pateclizumab is now in progress.

**Anti-RANK/RANKL treatment**
In mice, treatment with a neutralizing anti-RANKL monoclonal antibody in collagen-induced arthritis resulted in amelioration of bone loss. Phase II clinical trials suggest that denosumab-mediated inhibition of RANKL in RA patients prevents bone loss at the site of
inflammation, but has no apparent effect on inflammation 175. Nevertheless, denosumab could be used as an adjunctive therapy next to established (biologic) DMARDS to prevent structural joint damage in RA.

One has to bear in mind that virtually all stimuli that activate TNF receptor superfamily-members and induce non-canonical pathway signaling, simultaneously also activate the canonical pathway. Hence, determining whether the observed therapeutic effects of blocking these receptor-ligand pairs are mediated via inhibition of the non-canonical pathway or can also be attributed to effects on canonical NF-κB signaling is difficult and requires further investigation.

Future perspective: direct targeting of intracellular signaling molecules

Rather than indirect inhibition of non-canonical NF-κB signaling using the compounds described above, direct inhibition of NIK or IKKα may be more effective. In RA synovial inflammation, NIK can be targeted using intra-articular gene therapy (Noort et al., unpublished, ongoing studies in preclinical models of arthritis) or using small molecule inhibitors. Since NIK levels in normal cells are usually low, it is anticipated that therapeutics designed to limit the amount of NIK will not cause serious side effects. Consequently, NIK inhibition using specific small molecule inhibitors could perhaps be an effective treatment option not only for RA, but also for other chronic inflammatory diseases. Staurosporine is a pan-kinase inhibitor that has been demonstrated to inhibit NIK. However, due to its promiscuous nature it is unsuitable for clinical applications (reviewed in 176). Recently, a report was published, in which the discovery, structure-based design, synthesis, and optimization of several selective NIK inhibitors are described 177. The recent description of the crystal structure of the catalytic domain of NIK may further facilitate the development of new potent and selective NIK inhibitors 178,179. So far, only one specific IKKα inhibitor, BAY32-5915, has been reported 180. We expect that preclinical studies will soon answer the question whether selective targeting of NIK or IKKα and consecutive non-canonical NF-κB signaling may be beneficial in (rheumatoid) arthritis as well. Alternatively, in some cell types it may be beneficial to actually stimulate non-canonical NF-κB activity to obtain a therapeutic effect. Smac mimetics or inhibitor of apoptosis protein (IAP) antagonists that promote degradation of cIAP1 and cIAP2 are not only able to induce apoptosis in cancer cells, but also promote stabilization of NIK and consequent activation of the non-canonical NF-κB pathway in resting cells 181,182. Currently, one of these smac mimetic compounds called Birinapant is undergoing clinical development for the treatment of solid tumors and hematological malignancies 183,184.

CONCLUSIONS

The studies reviewed above support the role of the non-canonical NF-κB pathway as a major participant in inflammatory responses in general and the pathogenesis of RA in particular. NIK and downstream non-canonical NF-κB signaling has diverse functions, depending on the cell types in which this pathway is activated. Roughly, in synovial
fibroblasts, osteoclasts, endothelial cells, and B cells/plasma cells non-canonical NF-κB signaling contributes to the inflammatory process by triggering the secretion of critical inflammatory mediators and matrix degrading enzymes involved in tissue degradation, whereas in macrophages, dendritic cells and T cells this pathway has more pleiotropic functions and can also play a more regulatory, anti-inflammatory role (Figure 2). In these cells non-canonical NF-κB signaling shows clear resemblance with “The Strange Case of Dr Jekyll and Mr Hyde” (Robert Louis Stevenson): there is a good and an evil side. Further elucidation of the precise function of this pathway in individual cell types will result in a better understanding of the overall contribution role of the non-canonical NF-κB pathway to the complex cellular networks involved in RA synovial inflammation. This will allow the design of better therapeutic strategies for the management of this disease, including cell type-specific inhibitors or selective targeting of inhibitors to certain cell types. For this application anti-DEC-205 antibodies may be used to target compounds specifically to dendritic cells or to selectively target endothelial cells, (peptide) inhibitors can be coupled to a multimodular recombinant protein that specifically binds to cytokine-activated endothelium, which has been demonstrated to work very elegantly under inflammatory conditions in vivo.

Figure 2. Non-canonical NF-κB signaling in different cell types in RA: Dr. Jekyll or Mr. Hyde? Schematic overview of the role of the non-canonical NF-κB pathway in cell types involved in the pathogenesis of RA. On the left side (Dr. Jekyll), cells in which non-canonical NF-κB signaling plays an anti-inflammatory role. In the middle (on dashed line), cells in which this pathway plays a dual role which is largely dependent on the microenvironment. On the right side (Mr. Hyde), cells in which non-canonical NF-κB signaling acts pro-inflammatory.
Although the non-canonical NF-κB pathway has anti-inflammatory effects in some cell types, we believe that in chronic inflammation the positive effects of targeting the non-canonical NF-κB pathway will surpass the possible negative effects. We anticipate that the advent of several selective NIK inhibitors will aid in further establishing the non-canonical NF-κB pathway as a promising new therapeutic target, not only in RA, but also in other immune-mediated inflammatory diseases.

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