Targeting NF-kB to modulate immune responses in arthritis
Tas, S.W.

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Rheumatoid arthritis (RA) is a chronic autoimmune disease, characterized by painful, swollen joints and disability. Although treatment of this disease has been improved over the years, not all patients respond well to current treatment regimens. Ideally, new therapies should specifically block the inflammatory response in the joint, while normal immune responses to pathogens remain intact and side-effects are limited. Specific targeting of intracellular signal transduction intermediates involved in the initiation and perpetuation of synovial inflammation appears to be an elegant and powerful way to combat the pathological cellular processes observed in arthritis. In RA synovial tissue the NF-κB family of transcription factors is highly activated and can induce transcription of pro-inflammatory cytokines, adhesion molecules and other (destructive) factors, thereby contributing significantly to the RA disease process. Therefore, selective targeting of NF-κB may inhibit synovial inflammation and protect against joint destruction. This thesis focuses on the feasibility of NF-κB inhibition as a therapy for RA, using both gene therapy and small molecule inhibitors. In addition, the presence of dendritic cell (DC) subsets in RA synovial tissue is demonstrated. Finally, the consequences of NF-κB inhibition in DC are investigated, as an initial step towards the development of cell-based immunotherapy for RA.
Targeting NF-κB to modulate immune responses in arthritis

SANDER WILHELMUS TAS
CIP-GEVESENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Tas, Sander Wilhelmus

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Targeting NF-κB to modulate immune responses in arthritis

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Ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam op gezag van de Rector Magnificus prof. mr. P.F. van der Heijden ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Aula der Universiteit op woensdag 12 april 2006, te 14.00 uur

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Sander Wilhelmus Tas

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Promotor: Prof. dr. P.P. Tak

Overige leden: Prof. dr. L.A. Aarden
Prof. dr. W.B. van den Berg
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Faculteit der Geneeskunde, Universiteit van Amsterdam
“I was just guessin’, at numbers and figures, pullin’ the puzzles apart”

(*Coldplay, The Scientist*)

Aan mijn ouders
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Chapter 1

General Introduction
RHEUMATOID ARTHRITIS: CLINICAL AND HISTOPATHOLOGICAL FEATURES

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by persistent joint swelling and progressive destruction of cartilage and bone. The primary manifestations are pain, swelling, and limited motion of joints due to inflammation of the synovial membrane. Some patients may experience a mild illness, but in the majority of the patients the disease leads to the development of joint destruction, deformity and disability. RA is still associated with long-term morbidity and early mortality despite treatment with anti-rheumatic drugs (reviewed in (1)). In RA the synovial tissue is characterised by pronounced angiogenesis; cellular hyperplasia; influx of inflammatory leukocytes; and changes in the expression of cell-surface or adhesion molecules, matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), and a variety of cytokines. The synovial cell infiltrate comprises high numbers of activated macrophages, plasma cells, and T cells, in addition to fibroblast-like synoviocytes (FLS), dendritic cells (DC), B cells, natural killer cells and mast cells that perpetuate the inflammation (reviewed in (2,3)). CD4 positive T cells become activated when the T cell receptor recognises fragments of auto-antigens presented by the MHC class II molecules of antigen presenting cells (APC), like monocytes, B cells and DC. This may contribute to the initiation and perpetuation of the synovial immune response.

Current RA treatments are largely empirical in their origin and their precise mechanism of action is unknown. Increasing evidence shows that pro-inflammatory cytokines play a key role in chronic inflammatory diseases, including RA. This view is supported by the recent success of systemic targeted therapies, in particular TNFα blockade. It is now possible to reach at least 20% improvement in about 60–70% of the RA patients using this approach (reviewed in (4)). The majority of these patients, however, will still have some actively inflamed joints. In addition, almost 30% of the patients do not respond at all to TNFα blockade with regard to arthritis activity. In these patients, particularly when isolated arthritis activity exists despite systemic disease-modifying antirheumatic therapy, there is an indication for local treatment. Corticosteroids are widely used for this purpose, but not all patients respond to the use of intra-articular corticosteroids and its use is limited by side effects after prolonged treatment (5,6). Therefore, more specific local inhibition of inflammation is required with less side effects. Specific targeting of signal transduction pathways or transcription factors could be an elegant and powerful way to interfere with pathological cellular processes seen in arthritis.
THE ROLE OF NF-κB IN RA

Nuclear factor (NF)-κB is regarded as one of the most important pro-inflammatory transcription factors and plays an essential role in the transcriptional activation of interleukin (IL)-1 and TNFα (7). Conversely, NF-κB activation is also induced by these and many other pro-inflammatory stimuli, resulting in a positive feedback loop that may maintain or amplify inflammation. Thus, NF-κB, which can be activated via different NF-κB signal transduction pathways, plays a key role in a wide range of inflammatory diseases.

The canonical (also known as: classical) NF-κB pathway requires activation of the inhibitor of κB (IκB) kinase (IKK) complex, consisting of the catalytic subunits IKKα and IKKβ (8,9), and the regulatory subunit NEMO/IKKγ (10,11), followed by IKK-mediated degradation of the inhibitory IκB proteins. This results predominantly in the activation and nuclear translocation of the classical NF-κB dimer p50-RelA (Figure 1). In this pathway IKKβ is essential for NF-κB activation in response to pro-inflammatory stimuli like LPS, TNFα and CD40L (12-15), whereas

![Diagram of NF-κB signal transduction pathways]

**Figure 1. Schematic representation of the NF-κB signal transduction pathways.** Nuclear factor-κB (NF-κB) can be activated by a multitude of different stimuli, like TNFα, LPS and CD40L, but also via T cell receptor (TCR) signaling. Activation of the canonical (also known as classical) pathway depends on the IKK complex, which is composed of the kinases IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO). Activated IKK phosphorylates (P) IκBα to induce its degradation by the 26S proteasome, allowing NF-κB dimers (p50-p65) to translocate to the nucleus and bind to DNA to induce NF-κB target gene transcription. Activation of the non-canonical (also known as: alternative) pathway is strictly dependent on IKKα homodimers. The target for IKKα by NIK is phosphorylated and incompletely degraded into p52, resulting in the release and nuclear translocation of p52-RelB dimers. This pathway can be triggered by the activation of members of the TNF-receptor superfamily such as CD40L (that also induce canonical NF-κB signaling), but not via pattern recognition receptors such as TLR4.
IKKα is dispensable for IKK activation and induction of NF-κB DNA-binding activity in most cell types (15-17).

In contrast, the non-canonical (also known as: alternative) pathway is strictly dependent on IKKα homodimers and does not require IKKβ and NEMO/IKKγ (18,19). The target for IKKα homodimers is NF-κB2/p100, which is incompletely degraded into p52 upon activation of IKKα by NF-κB-inducing kinase (NIK), resulting in the release and nuclear translocation of p52-RelB dimers (Figure 1). This pathway can be triggered by the activation of members of the TNF-receptor superfamily such as the lymphotoxin β receptor, B-cell activating factor belonging to the TNF family (BAFF)-receptor and CD40L (that also induce canonical NF-κB signaling), but not via pattern recognition receptors such as Toll-like receptor 4 (TLR4), the receptor for LPS (20). It has been suggested that the canonical and non-canonical NF-κB pathways play distinct roles in immunity (reviewed in (21)). Recent literature implies a role for the non-canonical pathway in the regulation of immune responses, as IKKα is implicated in the negative regulation of inflammation (22) and NIK has a role in the development of regulatory T cells (Treg)(23).

In RA synovium NF-κB is highly activated and can induce transcription of pro-inflammatory cytokines, adhesion molecules and inducible nitric oxide (24,25), thereby contributing significantly to the RA disease process. Animal models of inflammatory arthritis also support the notion that NF-κB activation plays a pathogenic role in vivo. This has been shown in rats with streptococcal cell wall-induced arthritis (26) and adjuvant arthritis (27), and in mice with collagen-induced arthritis (28,29). Phosphorylation of IκB proteins is an important step in NF-κB activation via the canonical pathway and is induced by IKK. Blocking NF-κB through overexpression of IκBα by adenosviral gene transfer results in induction of apoptosis in the synovium with concomitant clinical improvement in rats with arthritis (26). IκBα gene transfer in human synovial tissue culture reduces spontaneous production of pro-inflammatory cytokines without an effect on anti-inflammatory mediators (30). Moreover, IκBα overexpression has been demonstrated to inhibit the production of MMP-1 and MMP-3, without affecting tissue inhibitor of MMP (TIMP-1). It is as yet unclear which proportion of MMP expression can be inhibited by blocking NF-κB, since other pathways like MAPK leading to AP-1 activation are also involved in the upregulation of matrix degrading enzymes in RA (30).

IKKβ, but not IKKα, is the target for pro-inflammatory stimuli in RA FLS, resulting in NF-κB activation and prevention of apoptosis (31,32). Recently, it has been demonstrated that blocking NF-κB by adenosviral gene therapy with a dominant negative IKKβ mutant ameliorates rat adjuvant arthritis, whereas activation of NF-κB by wild type IKKβ gene transfer induces arthritis in normal rats (27). In addition, a study was conducted to link IKKβ expression in human RA synovial tissue with NF-κB activity, erosive vs. non-erosive disease, and progression of joint
destruction (J. Ludikhuize et al. *Manuscript in preparation*). Using immunohistochemical staining procedures it was demonstrated that IKKβ is also highly expressed in human RA synovium and correlates significantly with NF-κB activation as measured by the expression of phosphorylated (ph) IκBα (r=0.83 Pearson correlation coefficient; R²=0.69) (Figure 2). Furthermore, high expression of IKKβ and phIκBα correlated with erosive disease (p<0.05) and radiological progression of joint destruction after 1 year (p<0.05). These findings support the view that IKKβ plays a central role in the pathogenesis of arthritis. Therefore, we propose that canonical NF-κB activation contributes to synovial inflammation and that selective targeting of IKKβ may inhibit synovial inflammation and could perhaps protect against joint destruction.

**STRATEGIES TO INHIBIT NF-κB LOCALLY IN THE JOINT**

**Gene therapy**
A particularly interesting approach could be to use intra-articular gene therapy to target IKKβ locally in the inflamed joint in order to have a therapeutic protein synthesized at the site...
of inflammation, without the potential dangerous side effects of systemic NF-κB inhibition. Gene therapy presents an attractive treatment option for RA, as the synovial compartment is the key target of the disease and most joints affected are easily accessible. Viral-mediated gene transfer is currently the most efficient system for delivering therapeutic proteins in vivo (33). Although gene therapy with adenoviral vectors has proven to be efficient for target validation in animal models of arthritis, this approach might not be feasible to treat patients until safety issues and the immune response to the vectors have been resolved. Adenoviral vectors may evoke serious host immune responses and as a consequence give only transient expression of the transgene (34).

Therefore, novel vectors have been developed and tested for their transduction efficiency as well as the stability and duration of transgene expression. We and others have investigated the use of adeno-associated vectors (AAV) for gene therapy in RA (35). AAV has emerged as a potential novel vector that lacks many of the immunogenic characteristics of adenoviral vectors and appears to be safe (36). AAV is a single-stranded DNA virus that does not induce a significant immune response and is not associated with disease in humans. rAAV vectors are particularly useful in targeting slowly dividing cells, which gives them great potential for the treatment of chronic disease because of their capacity to mediate long-term transgene expression. At present, rAAV is considered the most promising viral vector for gene therapy in RA (33).

Small molecule inhibitors

Although gene therapy for RA is very promising, there is also a lot of attention for the development of specific small molecule inhibitors of signal transduction. Recently, new technology, kinase-specific libraries and structure-based drug design, together with high-throughput screening, has led to the discovery and development of new compounds that inhibit specific kinases and selectively interfere with signal transduction pathways (37,38). At present, many of these compounds are extensively studied in animal models of specific diseases and also tested in healthy volunteers for their safety profile. However, it can be envisioned that systemic NF-κB blockade may lead to intolerable toxicity. Therefore, we aim at blocking NF-κB activity locally in the inflamed joint using new, specific peptide inhibitors that selectively target IKKβ and largely lack immunogenicity.

THE ROLE OF DENDRITIC CELLS (DC) IN RA

Currently, increased interest exists in cell-based therapies for inflammatory disorders, like stem cell transplantation (39), transduction of autologous cells directly in vivo or ex vivo using gene therapy (33), or ex vivo manipulated tolerizing dendritic cells (DC) to induce remission
or immunoregulation of disease (40-43). DC are crucial regulators of the immune response to foreign and self-antigens with many unique and powerful features (reviewed in (44,45)). DC are the most important APC for naive T cells. Since autoimmunity is regarded to result from the presentation of autoantigens by APC leading to an unwanted immune response, DC are thought to play crucial roles in both the initiation and the perpetuation of synovial inflammation in RA. It has been demonstrated that DC infiltration into affected tissue occurs early in disease pathology and that DC are enriched both in the synovial tissue and synovial fluid of affected joints (46-48). In addition, the inflamed synovial tissue of RA patients contains fully matured DC expressing high levels of class I/II MHC and T cell costimulatory molecules, clustered around activated T cells (47,49-51), suggesting that presentation of arthritogenic peptides to naive T cells may result in activated autoreactive T cells (52). Experimental evidence comes from a study showing that arthritis could be induced in DBA/1 mice by transfer of ex vivo collagen-pulsed DC (53). Although the autoantigen(s) involved in the pathogenesis of RA are still unknown, fully mature DC that present candidate autoantigens like type II collagen and cartilage glycoprotein 39 can be found in RA synovial tissue (54-56). Moreover, these DC may also activate newly infiltrated T cells and this may be sufficient to drive organ inflammation and disease. In view of these observations, we propose that synovial DC are not only crucial for (auto)antigen capture leading to autoimmunity, but also have a crucial role in sustaining established inflammation. Disruption or alteration of the interaction between DC and T cells in arthritis proves to be beneficial as intra-articular injection of an adenoviral vector containing CTLA4Ig ameliorated the disease in a murine model of type II collagen-induced arthritis. These antibodies prevented the interaction between DC and T cells leading to a delayed onset and a reduced severity of arthritis which lasted up to 20 weeks (57). Several human studies also showed that costimulatory molecule blockade in RA patients by intravenous treatment with CTLA4Ig is beneficial (58-60).

**NF-κB INHIBITION IN DC AS A TOOL TO MODULATE IMMUNE RESPONSES**

Through their exclusive ability to instruct naive T cells DC are capable of inducing both T cell proliferation as well as anergic or regulatory T cells in response to antigens (61,62). It is well established that the transcription factor NF-κB, in addition to its key role in mediating inflammatory responses in general (7), also has an important role in the regulation of DC development, maturation and APC function (63). NF-κB activation controls both the expression of co-stimulatory molecules and MHC, as well as the production of cytokines like IL-12 (64). Therefore, NF-κB has a major influence on all signals that are involved in DC-mediated T cell activation. Consequently, inhibition of NF-κB activity results in a reduced capacity of DC to secrete pro-inflammatory cytokines and to stimulate naive CD4+ and CD8+ T cells (reviewed in (62)). This information could potentially be applied to utilize DC for changing unwanted cel-
lular immune responses in auto-immunity, allergy or after transplantation through the induction of tolerance. T cell tolerance by DC can be achieved in three ways: 1) the induction of T cell anergy; 2) deletion of T cells via apoptosis; and 3) induction of regulatory T cells (reviewed in (65)). One of the most powerful ways to achieve tolerance is via canonical NF-κB inhibition in DC (66). The role of the non-canonical pathway in DC has not been fully characterized. Recent studies in macrophages suggest a role in the control of inflammation, through IKKα-mediated accelerated turnover of RelA and c-Rel, and their removal from pro-inflammatory gene promoters (22) or IKKα-mediated regulation of IKKβ kinase activity (67).

Thus, canonical NF-κB appears to be an important mediator of antigen-induced T cell activation, and specific canonical NF-κB inhibition in DC cells might block activation and proliferation of T cells or induce regulatory T cells, resulting in regulation of the immune response. Additional proof for this hypothesis has recently been provided by a study showing that NF-κB inhibition in DC by adenviral transfer of its endogenous inhibitor IkBα resulted in reduced expression of MHC class II and costimulatory molecules together with less pro-inflammatory cytokine production in vitro (68). Adenoviral transfer of a dominant negative IKKβ mutant to DC resulted in blockade of CD40L-induced NF-κB activation, cytokine production and up-regulation of costimulatory molecules and MHC class II, leading to reduced T cell stimulation (69). Therefore, we aim to specifically block canonical NF-κB activation in DC and investigate whether these DC acquire a tolerogenic or regulatory phenotype that would make them suitable as a (future) tool to modulate immune responses in arthritis via cell-based immunotherapy.

OUTLINE OF THIS THESIS

Section I is focussed on the validation of NF-κB, and more specifically IKKβ, as a therapeutic target in animal models of arthritis.

Chapter 2 discusses the major signal transduction pathways involved in the pathogenesis of RA and the advances in targeting a number of key intracellular pathways, including the NF-κB pathway. In addition, recently identified lead molecules and selected compounds under development for the treatment of inflammatory diseases are discussed.

IKKβ is a key regulator of synovial inflammation and it has been demonstrated that intra-articular introduction of a dominant negative form of IKKβ using adenviral gene therapy significantly reduced arthritis activity (27). Extensive study of synovial tissue may contribute to a better understanding of the mechanisms via which potent immunomodulatory drugs or constructs exert their beneficial effect. In chapter 3 we describe immunohistochemical stain-
ing procedures for the evaluation of synovial inflammation in rat adjuvant arthritis. These procedures will be used throughout the research described in this thesis to evaluate the effects of new anti-arthritic therapies.

Gene therapy with adenoviral vectors has proven to be efficient for target validation in animal models of arthritis, but in light of safety issues rAAV is considered a more promising viral vector for gene therapy in RA patients. In chapter 4 we investigate the tropism of rAAV serotypes 1-5 for synovial tissue in order to establish which serotype can be used best for local gene therapy in arthritis. Chapter 5 demonstrates the feasibility and beneficial effects of local AAV-mediated gene therapy targeting IKKβ in arthritis.

Besides exploring the possibilities of gene therapy, we also investigate the possible use of low-molecular compounds in RA. Therefore, we performed in vitro and in vivo experiments with several new small molecule NF-κB inhibitors. In chapter 6 the effects of local IKKβ inhibition in the joint using the NBD peptide are described.

Section II is focussed on the role of dendritic cells (DC) in arthritis and investigates the effects of NF-κB inhibition in DC, as an initial step towards the development of cell-based immunotherapy for RA.

In chapter 7 the characteristics of two major DC subsets, myeloid and plasmacytoid DC, in RA synovial tissue are described, by investigating their frequency, phenotype, distribution and cytokine expression.

The possibility to manipulate DCs in vivo or ex vivo using low molecular weight drugs, enabling them to exert tolerogenic activities, is increasingly studied (62). In chapter 8 the effects of the NF-κB inhibitor caffeic acid phenethyl ester (CAPE) on DC are investigated. Chapters 9 and 10 describe the effects of specific IKKβ inhibition in DC in vitro using the NBD peptide. Furthermore, chapter 10 demonstrates that the canonical and non-canonical NF-κB pathways have distinct roles in inflammation, in part via differential effects on the expression of the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO) and pro-inflammatory cytokines.

In chapter 11 the main findings of the studies in this thesis are summarized and discussed in light of recent literature.
REFERENCE LIST


Chapter 2

Signal Transduction Pathways and Transcription Factors as Therapeutic Targets in Inflammatory Disease: Towards Innovative Antirheumatic Therapy

Sander W. Tas, Philip H.J. Remans, Kris A. Reedquist and Paul P. Tak, M.D., Ph.D.

Div. of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

ABSTRACT

Many chronic inflammatory diseases are associated with deregulated intracellular signal transduction pathways. Resultant pathogenic interactions between immune and stromal cells lead to changes in cell activation, proliferation, migratory capacity, and cell survival that all contribute to inflammation. Increasing efforts are now being made in the design of novel therapeutic compounds to interfere with signaling pathways in inflammatory diseases like rheumatoid arthritis (RA). In this review we will outline the major signal transduction pathways involved in the pathogenesis of RA. We will assess advances in targeting a number of key intracellular pathways, including nuclear factor-κB (NF-κB), mitogen-associated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K)/Akt, signal transducers and activators of transcription (STATs), and reactive oxygen species (ROS) production. Finally, we will discuss recently identified lead molecules and the progress of selected compounds towards becoming new drugs for the treatment of inflammatory diseases.
INTRODUCTION

Specific targeting of individual signal transduction molecules could be an elegant and powerful way to interfere with pathological cellular processes in which signaling cascades have become deregulated and are either autonomously activated or selectively inhibited. Gaining insight into the mechanisms involved in the regulation of signal transduction pathways has become increasingly important in understanding cellular behavior both in normal biology and in pathological situations. Under physiologic conditions there is constitutive low-level activation of numerous signaling pathways that have regulated cross talk and can immediately respond to a range of stimuli to effectively adapt to new situations. This is a quick, highly regulated and dynamic process that is essential for cell survival and normal function (1). Especially when a cell encounters danger signals, such as stress, micro-organisms or toxins, intracellular signaling cascades are crucial to respond properly to the threats posed on the organism. Although individual pathways transmit signals along linear tracts, it has become apparent that these linear pathways are not free standing entities, but part of an increasingly complex network of interconnected cascades.

Signaling components can be activated through a variety of critical upstream regulators, and in their turn transmit a signal to a multitude of effectors. Although many signaling molecules are widely expressed in tissues and the overall function of these components and the logic of circuitry seem to be preserved from cell type to cell type, interference in a single pathway may lead to different effects in different cell types.

Various pathological conditions like autoimmune diseases are associated with high-level autonomous or induced activation of one or more signal transduction pathways, interfering with normal cell biology and leading to pathogenic interactions with other cells. In this review we will focus on rheumatoid arthritis (RA) as a prototype of inflammatory disease.

RA is characterized by chronic inflammation of the synovial tissue in the joints leading to joint destruction (2). In the synovial tissue of RA patients, striking histologic changes are found (reviewed in (3)). Normal synovium is composed of one to three cell layers. The intimal lining layer contains fibroblast-like synoviocytes and intimal macrophages. Underneath the intimal lining layer is the sublining, containing blood vessels, nerves, and a small number of fibroblasts, adipocytes, macrophages and lymphocytes (4). In rheumatoid synovium the intimal lining layer shows marked hyperplasia, mainly due to expansion of intimal macrophages and fibroblast-like synoviocytes (5). The fibroblast-like synoviocytes also display altered biology, characterized by anchorage-independent growth and resistance to apoptosis due to constitutive activation of multiple signaling cascades (reviewed in (6,7)).

The synovial sublining is characterized by an inflammatory infiltrate, predominantly consisting of numerous macrophages, T cells and plasma cells. Small numbers of other cell types, like B cells, natural killer (NK) cells, mast cells, neutrophils and dendritic cells (DC) are also present (8,9). Increased angiogenesis is observed, and vessels in the sublining transform
into high endothelial venules that are involved in recruitment of inflammatory cells (10).

In active stages of RA, marked edema and fibrin deposition are present as well (11,12). In the pathogenesis of RA many cell types are involved that play distinct but important roles. Macrophages appear to play a pivotal role in the pathogenesis of RA as they are present in high numbers in RA synovial tissue and clearly show signs of activation including enhanced expression of cellular surface markers like MHC class II molecules, and production of pro-inflammatory cytokines like tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) (13), chemokines and matrix metalloproteinases (MMPs) (14). Also, a positive correlation was found between local disease activity, macrophage numbers and TNF\(\alpha\) expression in the synovial cell infiltrate (9). The efficacy of biological therapies directed against cytokines that are predominantly produced by these cells confirms the important role for macrophages in the perpetuation of the inflammatory process in the joint (15).

Many CD4+ and CD8+ T cells that are either clustered in perivascular aggregates or scattered throughout the synovial tissue can be found in RA synovium. The CD4+ T cells are predominantly T helper 1 (Th1) cells that could aggravate the proinflammatory and destructive pattern associated with macrophage activation (16). Of interest, T cells in the synovial fluid and tissue show hypo responsiveness to proliferative stimuli, which is associated with a high rate of reactive oxygen species (ROS) production and deregulated signal transduction pathways in these cells (17,18).

B cells are found in the lymphocyte aggregates, which bear resemblance to germinal centers. These regions are surrounded by fields of plasma cells that have the capability to produce autoantibodies, including rheumatoid factors. Promising data with B cell directed therapy (anti-CD20 monoclonal antibodies) in RA suggest a key role of B cells in the pathogenesis of this disease (19,20).

The role of NK cells in RA is unclear. The presence of activated NK cells expressing granzyme B in RA patient synovial tissue positively correlates with serum levels of acute-phase reactants (21). A recent study reported that a subset of NK cells was expanded within inflamed joints. After isolation, these cells responded to interleukin (IL)-12 and IL-15 stimulation by rapidly secreting interferon-\(\gamma\) (IFN\(\gamma\)). This cytokine can activate macrophages, amplifying the production of proinflammatory cytokines within the joint (22).

Activated mast cells are present in rheumatoid synovium, at sites of cartilage destruction, and in synovial fluid (23). Because mast cells contain potent inflammatory mediators, including histamine, heparin, proteinases, leukotrienes and multifunctional cytokines, they may contribute to the processes of inflammation and matrix degradation, either directly or via the effects of these mediators on adjacent cells (24). In patients with RA, synovial mast cells express significant amounts of the complement receptor C5a (C5aR/CD88) and release histamine in response to complement component C5a. Taken together, this indicates a role for C5a/C5aR in the pathogenesis of RA and suggests that C5a blockade might be beneficial in RA (25). A recent experimental study demonstrated that development of joint inflamma-
tion was critically dependent on the presence of mast cells, which function as a cellular link between autoantibodies, soluble mediators, and other effector populations in inflammatory arthritis (26).

DC are likely to play an important role in the initiation phase of the disease as they are the most important antigen presenting cells for naive T cells and autoimmunity is regarded to result from the presentation of autoantigens leading to an unwanted immune response. Experimental evidence for this comes from a study that showed that arthritis could be induced in DBA/1 mice by transfer of ex vivo collagen-pulsed DC (27). Furthermore, in RA synovial tissue fully mature DC that present candidate autoantigens like type II collagen and cartilage glycoprotein 39 can be found (28,29).

Few neutrophils are found in RA synovial tissue, but they are abundantly present in RA synovial fluid. Here, neutrophils may contribute to cartilage destruction by the production of oxygen free radicals (30). Destruction of cartilage and bone in RA is also mediated by locally invasive tissue found at the junction between synovium and bone, called pannus. It is derived from synovium and consists of cells that share some characteristics with tumor cells like uncontrolled cell division and the ability to invade adjacent tissue (31). Lymphocytes, fibroblast-like synoviocytes expressing MMPs, and osteoclasts are found in the pannus (32,33). Osteoclasts play a crucial role in the pathogenesis of bone erosions. Sites typical of osteoclastic activity have been demonstrated by electron microscopy in areas of erosion of subchondral bone in metacarpal heads from patients with RA (34). Co-culture experiments using RA synovial fibroblasts demonstrated that osteoclast-like cells could be generated from peripheral blood mononuclear cell precursors (35). Additional studies showed that synovial tissue provides a source of receptor activator of NF-κB ligand (RANKL) that, in addition to its required role in the differentiation of osteoclasts from their precursor cells, also could augment osteoclast activity and survival leading to bone destruction (reviewed in (36)).

Together, all the changes in cell activation, proliferation, migratory capacity, and cell survival could contribute to the inflammation and joint destruction in RA. Explaining these pathological changes, both in micro architecture and physiology of the synovium, is key to understanding the pathogenesis of RA. It is not unexpected that elucidation of signal transduction pathways has gained attention in attempts to understand the pathophysiological processes underlying RA and other chronic inflammatory diseases.

During the last ten years new technology, kinase-specific libraries and structure-based drug design, together with high-throughput screening, has led to the discovery and development of new compounds that inhibit specific kinases and selectively interfere with derailed signal transduction pathways (37,38). These tools have greatly facilitated research in this complex field, not only by allowing us to obtain more insight into the role of individual signal transduction molecules involved in the pathogenesis of RA, but they also offer the prospect of discovering new therapeutic targets. In this review we will outline the changes in major signal transduction pathways that are associated with RA, the cell types involved,
and existing as well as promising (future) therapeutic strategies to correct or interfere with these pathways in such a way that they result in amelioration of arthritis or even restoration of normal homeostasis.

NUCLEAR FACTOR-κB

The transcription factor nuclear factor-κB (NF-κB) appears to be a key regulator of inflammation in RA. The mammalian NF-κB/Rel family has many members: RelA (p65), NF-κB1 (p50; p105), NF-κB2 (p52; p100), c-Rel and RelB. These proteins share a highly conserved nuclear localization sequence (NLS), as well as a DNA binding and a dimerization domain. c-Rel, RelB and RelA also have a transactivation domain that is necessary for transcription from NF-κB-binding sites in target genes. Each member, except for RelB, can form homodimers, as well as heterodimers with each other. The dimeric structure of NF-κB allows many different combinations to form, each exerting a distinct biologic function (reviewed in (39)). The main activated form of NF-κB is a heterodimer, consisting of a p50 or p52 subunit and the transactivating subunit p65.

Inactive NF-κB resides in the cytoplasm associated with regulatory proteins called inhibitors of κB (IκB), of which IκBα, IκBβ and IκBε may be the most common. Importantly, the precursor proteins p100 and p105 can also function as IκB-like proteins. For many years it was thought that IκB proteins exert their function by masking the NLS on NF-κB subunits. However, recent studies revealed that only one of the two NLS in a NF-κB dimer is masked by IκBα, allowing the complex to shuttle to the nucleus (40,41). The nuclear export signal on IκBα functions to expel the NF-κB-IκBα complex from the nucleus (42). Because the export process is more efficient than the import process, the net result of this continuous movement between nucleus and cytoplasm is cytoplasmic localization of inactive NF-κB. The biologic implications of this energy consuming constant shuttling of NF-κB-IκBα complexes are currently unclear and need further investigation.

IκBα regulates transient NF-κB activation, whereas IκBβ is involved in sustained activation. Different IκB proteins have distinct and overlapping specificities for NF-κB proteins and tissue distribution of IκBs may also differ, making them attractive targets for specific therapies (43).

For most known stimuli, degradation of IκBα is essential for release and activation of NF-κB. Phosphorylation of IκB at specific serine residues by the IκB kinase (IKK) complex and subsequent polyubiquitination by β-TRCP targets IκB for degradation by the 26S proteasome, releasing NF-κB dimers from the NF-κB-IκB complex, followed by translocation to the nucleus and binding to κB enhancer elements of target genes.

The IKK complex contains at least two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and the regulatory subunit IKKγ (or NEMO, NF-κB essential modulator) that acts as a scaffold (44). Multiple signaling pathways that lead to NF-κB activation, like TNFα signaling via TRAF2/RIP/
MEKK3, Toll-like receptor (TLR) signaling via IRAK/TRAF6/NIK/TAK1 and T cell receptor (TCR) signaling via PKCθ/MAGUK/BCL10, converge at the level of the IKK complex (Figure 1). IKKα and IKKβ have very distinct functions in vivo. IKKβ is essential for activation of IKK by TNFα and IL-1, making it the most important kinase for IκB degradation and NF-κB activation in inflammatory conditions like RA (45-47). IKKα has a role in enhancing the transactivation function of NF-κB independent of IκB degradation, possibly by regulating chromatin structure at promoter sites via histone phosphorylation (48,49). Furthermore, IKKα also plays an important role in the non-canonical NF-κB pathway that regulates for instance RANKL signaling, which is involved in bone remodeling, DC-T cell communications and lymph node formation (50,51). Although IKKγ/NEMO does not have kinase activity, new data indicate that in addition to its key role in regulating cytokine-induced IKK activity, IKKγ is also able to shuttle between the cytoplasm and nucleus, and can lead to transcriptional repression of the NF-κB pathway by binding to CREB-binding protein (CBP) (52).

Regulation of transcriptional NF-κB activity does not only take place at the level of the IKK complex, but also via direct modification of NF-κB proteins through phosphorylation. IL-1 and TNFα induced phosphorylation of the NF-κB p65 subunit is mediated by pathways...
that are different from those that lead to IκB degradation and subsequent NF-κB activation. This phosphorylation of p65 at specific Ser positions is essential for efficient binding to the transcription co-activator CBP (53), enhancement of its transcriptional activity (54) and its transactivation function (55). Similar regulatory mechanisms have been described for the NF-κB p50 subunit, which exhibits increased NF-κB DNA binding activity after phosphorylation via IL-1-stimulated phosphoinositide 3-kinase (PI3K)/Akt (56).

Another mechanism by which transcription of NF-κB responsive genes can be regulated is via modification of histone acetylation by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation status influences the accessibility of DNA to the transcriptional machinery by changing the folding and functional state of the chromatin fiber (57). NF-κB interacts with HATs to positively regulate gene expression and with HDACs to negatively regulate transcription of NF-κB responsive genes (58,59). Recent reports indicate that acetylation and deacetylation of histones is a regulated process and can serve as a switch to control the duration of NF-κB transcriptional responses (59,60).

NF-κB induces the transcription of pro-inflammatory cytokines like IL-1β and TNFα in monocytes and macrophages (61-63), and IL-6 in fibroblast-like synoviocytes (64).

Transcription of chemokines (e.g. CCL2, CCL5, CXCL8, CXCL9 and CXCL10) (65) and the expression of cell adhesion molecules like E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 are also regulated by NF-κB (66,67), indicating an important role in leukocyte adhesion and transmigration.

Other functions include promoting the expression of cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) and MMPs. In many cell types (like fibroblast-like synoviocytes, neutrophils and T lymphocytes) NF-κB plays an anti-apoptotic role (68-70), which may be regulated via Akt, the suppression of caspase-8 activation or IKKβ related functions (45,71,72).

NF-κB also functionally interacts with other pathways and transcription factors, like activator protein 1 (AP-1) in synoviocytes and osteoclasts, to coordinate stimulation of MMP production leading to bone and cartilage destruction (73,74).

The role of NF-κB in RA
NF-κB plays a key role in the expression of pro-inflammatory genes and is abundant in inflamed tissues like rheumatoid synovium (43,75,76). In synovial tissue of RA patients, high levels of both p50 and p65 can be detected in synovial lining cells and sublining mononuclear cells (76), accompanied by high expression of pro-inflammatory mediators like cytokines and chemokines, and infiltrating immune cells. Macrophages are thought to be the main producers of TNFα in RA and this production is regulated by NF-κB (61). As referred to earlier, increased IL-6 production in fibroblast-like synoviocytes is also associated with constitutive NF-κB activation or IKKβ-mediated hyperresponsiveness to cytokine-induced NF-κB activation (47,77,78).
In RA synovial tissue, fully mature DC expressing high levels of MHC class I/II, T cell costimulatory molecules and nuclear RelB are found localized in clusters around activated T cells (28,79). These DC have been demonstrated to present auto-antigens like type II collagen and human cartilage glycoprotein 39, and prime T cells for a Th1-type immune response (28,80). In T cells NF-κB activity is required for normal TCR signaling and differentiation of naive T cells into a Th1 phenotype (81,82). NF-κB plays a crucial role in the regulation of B cell maturation and is also required for normal osteoclast development and function (32,83). In osteoclasts the TRAF6/NF-κB pathway is an important signal transduction route in RANK-RANKL mediated differentiation and activation of osteoclasts (reviewed in (84)), which play a key role in bone destruction in arthritis (85,86). Therefore, modulation of RANKL signaling provides a unique opportunity to design novel therapeutics to inhibit bone erosions and joint destruction in arthritis (reviewed in (51)).

In vivo evidence for the importance of NF-κB comes from numerous studies in animal models of arthritis. Early after induction of arthritis in both murine collagen induced arthritis (CIA) and rat adjuvant arthritis (AA) models, prominent NF-κB expression can be found in the intimal lining layer that precedes the development of clinical signs of arthritis (74,87).

**NF-κB inhibition**

Selective NF-κB activation by intra-articular transfer of a functional IKKβ gene is sufficient and essential to induce synovial inflammation and clinically manifest arthritis in normal rats (88). Conversely, intra-articular injection with a dominant-negative adenoviral IKKβ construct resulted in a reduction of NF-κB nuclear translocation and paw swelling in adjuvant arthritis (88). From these experimental data it can be concluded that targeting NF-κB at the level of IKKβ appears promising in inflammatory conditions like arthritis. Orally bioavailable IKK(β) inhibitors have been developed and tested *in vitro* and in animal models of arthritis. A selective IKKβ inhibitor, SC-154, was shown to block NF-κB dependent gene expression in IL-1β stimulated fibroblast-like synoviocytes *in vitro* and inhibited LPS induced serum TNFα production in rats (89). Another highly selective IKK inhibitor, BMS-345541, was shown to reduce both synovial inflammation and joint destruction in collagen -induced arthritis in mice (90), and treatment of arthritic rats with the orally bioavailable IKKβ inhibitor SPC839 resulted not only in amelioration of arthritis, but also in significantly reduced radiographic damage of the joints (91,92). Recently, the oral bioavailable IKKβ inhibitor MLN120B was demonstrated to dose-dependently decrease paw swelling in AA, which correlated with decreased NF-κB activity in the spleen (93).

Blocking the NF-κB pathway by the IκBα super-repressor resulted in suppressed constitutive and TNFα induced NF-κB activity and increased sensitivity to pro-apoptotic stimuli *in vitro*, both in normal macrophages and in RA synovial cell cultures and macrophages (61,69). Adenoviral vector -mediated IκBα overexpression inhibited the spontaneous production of TNFα and other pro-inflammatory cytokines in cultured explants of rheumatoid synovial
tissue and also inhibited the production of MMPs 1 and 3 while not affecting their tissue inhibitor (94).

Selective inhibition of NF-κB in T cells in transgenic mice overexpressing IκBα resulted in decreased incidence and severity of CIA (95). More evidence for the beneficial effects of NF-κB blockade in T cells comes from a study that used the T cell-specific NF-κB inhibitor SP100030 to systemically treat murine CIA, which resulted in significantly decreased arthritis severity (96). Its selectivity for T cells could theoretically provide potent immunosuppression with less toxicity than other non-cell type specific NF-κB inhibitors. Similarly, NF-κB blockade with NF-κB decoy oligonucleotides was shown to inhibit the development of arthritis in two rat models of arthritis (70,97). However, the therapeutic potential of decoy oligonucleotides is unclear as these molecules are quite large and polar, which will likely hinder the cellular uptake and bioavailability (98). NF-κB is redox sensitive, and for that reason small molecules that interfere with the redox potential could influence NF-κB DNA binding. This will be discussed later in this review in the section on reactive oxygen species (ROS).

NF-κB activity can also be blocked by small molecule inhibitors of the 26S proteasome, that reduce the degradation of IκB and thereby prevent NF-κB activation (99,100). Examples of these agents are a variety of peptide aldehydes that inhibit protease activity, a group of lactacystin based inhibitors that block protein degradation by acetylating a threonine residue in one of the key proteasome subunits, epoxyketones and 2-aminoazulines that inhibit the chymotrypsin-like activity of the proteasome, and finally a group of boronic acid peptides like PS-341 that blocks proteasome function very efficiently (101). In streptococcal cell wall-induced polyarthritis in rats, daily oral treatment with PS-341 resulted in considerable reduction of the inflammatory infiltrate. Moreover, there was only minimal degradation of the articular cartilage and markedly attenuated subchondral bone erosion (102). However, the 26S proteasome has many other important functions and inhibition of proteasome activity could potentially cause severe side effects.

As already alluded to earlier, most interest has focused on small molecule IKKβ inhibitors as they are more selective and might cause less severe side effects than other systemic approaches targeting NF-κB activity. Some of these inhibitors have been tested only in vitro, or in animal models for inflammatory diseases other than RA, but might be beneficial in arthritis as well. Examples include selective IKKβ serine-threonine protein kinase inhibitors (103), so-called β-carbolines that block IKK activity (104), and a NEMO -binding domain peptide that has been demonstrated to ameliorate peritonitis (105). Recently, some natural compounds and derivatives have also been demonstrated to possess IKK -inhibiting potential and might be beneficial in reducing the severity of arthritis (106-108). No potent IKKα inhibitors have been described to date, although the unique role in the alternative pathway for activation of NF-κB and its auxiliary role in the classical pathway indicate that IKKα might also be an attractive target in autoimmune diseases such as RA.
A possible approach for T cell specific NF-κB inhibition could be via targeting protein kinase C (PKC) θ, an essential kinase in T cell receptor mediated activation of NF-κB. This could be achieved by a recently developed PKC θ-inhibiting factor that proved to selectively inhibit T cell NF-κB activity in mouse models of contact hypersensitivity and delayed type hypersensitivity by blocking the PKC-PDK1 interaction (F. d'Acquisto and S Ghosh, personal communications).

Obviously, testing of these compounds in animal models of arthritis will allow more accurate conclusions to be drawn about their efficacy, safety, and therapeutic potential.

Finally, it is interesting to note that many of the drugs that are currently used for treating RA like non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and corticosteroids have effects on NF-κB activity. NSAIDs inhibit COX activity to prevent prostaglandin synthesis (109). However, aspirin, sodium salicylate and sulindac also function as competitive inhibitors of IKKβ to block NF-κB activation (110-112). DMARDs, such as sulfasalazine and leflunomide inhibit IκBα phosphorylation and subsequent degradation, thereby preventing nuclear translocation of NF-κB (113-115), and the widely used anti-rheumatic drug methotrexate is also thought to exert some of its effects via suppression of NF-κB activation (116). In addition, gold compounds have been demonstrated to inhibit the DNA-binding activity of NF-κB (117). Glucocorticoids have been proven to be one of the most potent drugs in reducing the severity of arthritis both in clinical and histologic parameters (118,119). They interact with the intracellular glucocorticoid receptor (GR) to downregulate the expression of specific genes that regulate inflammation (120). The effects of glucocorticoids on NF-κB activity take place at various levels: a) transcriptional activation of the IκBα gene to enhance cytosolic retention of inactive NF-κB (121,122), b) competition between NF-κB and the GR for limiting amounts of coactivators (123) and c) repression of p65 transactivation by GR mediated disruption of its interaction with the basal transcription machinery (124). Cyclosporine A inhibits activation of the transcription factors NF-κB and nuclear factor of activated T cells (NF-AT) by calcineurin (125). This drug also serves as a non-competitive proteasome inhibitor and thus prevents IκBα degradation and NF-κB activation (125,126).

It should be noted, however, that conventional DMARDs lack specificity for inhibiting NF-κB activity and consequently require relatively high concentrations, raising the issue of toxicity. Most of the targeted, more specific NF-κB inhibitors exert their action at the level of the IKK complex or IκBα and show promising therapeutic properties in animal models of arthritis. However, it is conceivable that with increasing understanding of the function of individual NF-κB subunits, IκB proteins, and kinases in different cell types and their contribution to the pathogenesis of RA, one might attain therapeutic efficacy with minimized systemic toxicity by selectively targeting the most important proteins and allow normal function of the other proteins. Although at present IKKβ seems to be the most promising target for NF-κB...
inhibition in arthritis, the ultimate benefit of such targeted therapy will probably be achieved by direct intra-articular approaches to limit the toxicity problems associated with systemic NF-κB inhibition.

**AP-1 inhibition**

Another key transcription factor involved in the pathogenesis of RA is activator protein (AP-1). This transcription factor is part of the bZIP family and regulates T cell activation, cytokine production and production of MMPs by various cell types. AP-1 proteins bind to DNA and activate transcription as Jun homodimers or Jun-Jun/Jun-Fos heterodimers (127). AP-1 is activated through the upstream mitogen-activated protein kinases (MAPKs) p38, JNK and ERK. All 3 MAPK pathways can regulate transcription and transcriptional activity of Fos and Jun family genes. They all contribute to up-regulation of c-fos gene transcription via Ets, but transcription of c-jun is primarily regulated via the JNK pathway and can be increased by c-jun itself via a positive autoregulatory loop (128,129). AP-1 activity is largely regulated via posttranslational modification of c-jun by phosphorylation. JNK1 and JNK2 can phosphorylate c-jun at both its 63 and 73 N-terminal serines in the transactivation domain and thereby dramatically enhance the inducible transcriptional activity of c-jun (129).

Indirect interference with AP-1 activation via targeting MAPKs will be discussed later in this review. Another way of inhibiting AP-1 activity is to directly interfere with its DNA binding capacity. Several natural product inhibitors of the fos-jun/DNA complex formation, like nordihydroguaiaretic acid (NDGA) and momordin I are available for this purpose (130). The effects of these compounds have as yet not been tested in arthritis models. As the transcriptional activity of AP-1 is redox sensitive, small molecules that interfere with the redox potential could influence AP-1 DNA binding. This will be discussed later in this review in the section on ROS.

**MAPK PATHWAYS**

MAPK pathways play a crucial role in many aspects of immune-mediated inflammatory responses. They represent a complex signal transduction network that integrates extracellular stress and pro-inflammatory stimuli (131). MAPKs play a central role in the regulation of immunological responses in general and in RA in particular.

MAPKs directly regulate certain transcription factors, like AP-1 and activating transcription factor 2 (ATF-2), and indirectly regulate other transcription factors, like NF-κB (132,133). One example of indirect regulation may be p38δα-dependent histone H3 phosphorylation that marks and recruits NF-κB to otherwise cryptic promotors, leading to increased transcription of several pro-inflammatory genes (134).
Activation of MAPK signal transduction pathways is regulated through phosphorylation of specific threonine and tyrosine residues within the activation loop by MAPK kinases (MAPKKs/MKKs or MEKs). These MEKs are in turn activated by upstream MEK kinases (MAPKKK/MAP3K/MKKK or MEKK) via phosphorylation at a pair of serine residues in the activation loop (135).

Three major MAPK pathways have been described: p38 kinases (p38α, p38β, p38γ and p38δ), c-Jun-N-terminal kinases (JNK1, JNK2 and JNK3) and extracellular signal-regulated kinases (ERK1 and ERK2). In general, ERKs are activated by mitogenic and proliferative stimuli like growth factors, while JNKs and p38 MAPKs respond to pro-inflammatory cytokines such as TNFα and IL-1 and to environmental stress, including ROS, ultraviolet irradiation, heat and osmotic shock (131). However, although the MAPK pathways have distinct functions, there is also considerable overlap and crosstalk between the p38, JNK and ERK pathways (Figure 2).

Figure 2. Schematic representation of the MAPK signal transduction pathways.
The family of mitogen-activated protein kinases (MAPKs) can be activated by different extracellular stimuli after receptor-ligand interactions. Stress/mitogens, cytokines and integrins can all induce phosphorylation (P) of multiple component MAPKK and MAPKK isoforms for each system. This leads to activation of one or more of the three main MAPKs: ERK, JNK and p38, which have distinct transcription targets. MAPKs can phosphorylate these transcriptional targets directly or this can occur via downstream protein kinases. Although the different MAPK pathways have distinct functions, there is also considerable overlap and crosstalk between the p38, JNK and ERK pathways.
TAK1 and MKK6, but recently it was reported that p38α is also activated by TAB1, which is not a MKK but an adaptor or scaffolding protein lacking clear catalytic activity (136). This indicates that regulatory mechanisms other than the MKKK-MKK-MAPK cascade exist. In addition, it indicates that other adaptor proteins should be analyzed for their potential role in regulating MAPK activity (137).

JNKs were first characterized as stress-activated protein kinases, because they were activated in response to inhibition of protein synthesis (138). Regulation of the JNK pathway is extremely complex and influenced by 13 MKKKs, allowing JNK activation by many different stimuli (reviewed in (137)). ERK1 and ERK2 are both components of a three-tiered kinase cascade usually initiated by Ras GTPase mediated activation of MKKK c-Raf1, B-Raf or A-Raf. This leads to phosphorylation of MKK1/2 and subsequent phosphorylation and activation of ERK1/2.

A diversity of regulated crosstalk exists both upstream and downstream of MAPK. Certain MEKKs can activate multiple MKKs leading to subsequent activation of both p38 and JNK MAPK pathways, as observed in most pro-inflammatory reactions (139,140). Downstream, MAPK activate dual specificity phosphatases, which can dephosphorylate and inactivate other MAPKs (141).

One important transcription factor regulated by MAPKs is AP-1, which is of importance in MMP and cytokine production. Another transcription factor that is regulated by MAPK is ATF-2, which is constitutively expressed and binds to DNA as a homodimer or a heterodimer with c-jun and other members of the bZIP family (142). ATF-2 transactivating potential is regulated via JNK1, JNK2 and p38 MAPK -mediated phosphorylation (143), leading to transcription of genes such as TNFα and various adhesion molecules (144-146). Thus, MAPK are extremely important in controlling the transcription of genes that encode pro-inflammatory factors, such as cytokines like IL-1, TNFα and IL-6, MMPs, adhesion molecules, COX2, and iNOS (131).

The role of MAPK in RA

There is an overwhelming body of evidence that MAPK play an important role in the pathogenesis of RA. Active phosphorylated forms of ERK, JNK and p38 have been detected in synovial tissue of RA patients (147). ERK is localized around synovial blood vessels, JNK around and within mononuclear cell infiltrates, and p38 MAPK activation can be observed in the intimal lining layer and in synovial endothelial cells (147). The expression of MAPKs in RA synovial tissue is paralleled by increased expression, nuclear localization and activity of AP-1, along with AP-1 regulated cytokines and MMPs (74,148).

In most inflammatory cells p38α is prominently activated and tightly associated with the production of inflammatory mediators that initiate leukocyte recruitment and activation. In synovial tissue activated p38 MAPK have been detected in endothelial cells and this could promote angiogenesis via activation of the Ets-1 transcription factor (149,150). Other effects of endothelial p38 MAPK activation include enhanced migration and accumulation of im-
mune cells by induction of E-selectin (151), VCAM-1 (152) and MCP-1 (153) expression, as well as iNOS-dependent vasodilatation (154).

The p38α isoform also plays a central role in TNFα signaling. It is involved both in TNFα-induced TNF-R signaling via TRAF6/TAK1/MKK6, and in macrophages, MAPKAP kinase-2–dependent TNFα production (155,156). Furthermore, cultured fibroblast-like synoviocytes from RA patients showed marked induction of p38, JNK and ERK activity together with accumulation of c-jun and c-fos messenger RNA in response to TNFα, IL-1 and IL-6 (147,157).

JNK-2 is constitutively expressed in RA fibroblast-like synoviocytes and appears to be the primary JNK isoform. In these cells JNK can be activated by pro-inflammatory cytokines and Fas ligand, and is involved in the activation of c-jun, which can induce MMP gene expression. The other MAPKs are also able to regulate MMP production, but their relative contributions depend on the cell type and the stimulus (158). A proportion of T cells in RA synovium express high levels of activated JNK. As JNK has been demonstrated to play a crucial role in Th1 cell differentiation and cytokine production, this may contribute to the shift towards Th1 cytokine expression profiles observed in RA (159,160).

Two MAPKK proteins act as upstream JNK activators, MKK7 and MKK4. MKK7 is primarily activated by cytokines and MKK4 is mainly activated by environmental stress like ultraviolet light (161). In RA synovium high levels of activated MKK4 and MKK7 are detected with a predominant localization in cells of the intimal lining layer, suggestive of expression in fibroblast-like synoviocytes. In vitro studies revealed that MKK4 and MKK7 activation by MEKK2 synergistically activate JNK in fibroblast-like synoviocytes after stimulation with cytokines, suggesting an important role for this MAPK pathway in RA (162). Immunoprecipitation studies showed that MKK4 and MKK7 co-precipitate with JNK, forming a complex referred to as the JNK signalsome, which may have a critical role in MAPK activation and expression of AP-1 regulated genes (91,162).

The role of ERK1/2 in RA is not completely understood. The primary role for ERK1/2-mediated signaling involves cell growth and proliferation, but it has become clear that several other processes involved in inflammation also require ERK activation. The ERK pathway is rapidly activated in RA fibroblast-like synoviocytes after brief exposure to IL-1 (163). In contrast to its role in fibroblast-like synoviocytes, ERK appears to be more important in articular chondrocytes, in which it is phosphorylated after IL-1 and TNFα stimulation. ERK activation in chondrocytes is even more pronounced after stimulation with LPS, PDGF, IGF and IL-6, agonists which do not activate JNK or p38 (164). Of interest, studies in knockout mice suggest that ERK activation is crucial for AP-1 mediated T cell activation, which might be important in activation of T cells in RA (165). Recently, the importance of continuous B cell receptor mediated self-antigen signaling to ERK was implicated in the inhibition of CpG DNA-induced plasma cell differentiation, indicating that ERK might have a protective role against Toll-like
receptor elicited autoantibody production and autoimmunity (166). In osteoblasts ERK activation suppressed the production of MMP13 induced by T cell cytokines (167).

**p38 inhibition**

Given the central role of MAPKs in inflammation, many different MAPK inhibitors have been developed and tested for their capacity to reduce inflammatory responses both *in vitro* and *in vivo*.

Because of the earlier described central role of p38α MAPK in the inflamed synovium and in light of the impressive clinical efficacy of anti-TNFα therapy, an orally active pharmacological agent that targets p38 and thereby inhibits the production and activity of pro-inflammatory cytokines could provide a promising treatment for RA.

p38 MAPK inhibitors have been developed by many pharmaceutical companies (Table 1). The compounds SB-203580 and SB-242235 were tested in AA in rats; both compounds ameliorated arthritis (168). SB-203580 and the further optimized compound SB-220025 were also beneficial in CIA in mice and reduced bone resorption (169,170). These compounds block the ATP-binding pocket of p38 and inhibit p38α and p38β more effectively than p38γ and p38δ.

Urea 22 reduced arthritis scores in CIA when mice were treated orally with this compound (171). RWJ-67657 and the selective imidazole L-167307 also showed efficacy in AA (172,173). Furthermore, a pyridinylloxazole-based inhibitor protected against inflammation in AA and CIA (174) and a series of 2-aminopyrimidines, RPR-200765A, RPR203494 and RPR-238677, displayed good oral anti-arthritis efficacy in streptococcal cell wall-induced arthritis in rats (175-177). Another potential anti-inflammatory drug, CNI-1493, which inhibits the phosphorylation of both p38 MAPKs and JNK (178-180), showed both protective and therapeutic effects in AA in rats (181,182).

FR167653, a potent inhibitor of TNFα and IL-1β production in stimulated monocytes and lymphocytes (183,184), both prevented AA in rats and suppressed joint destruction in established arthritis (185). Many other potent orally available p38 MAPK inhibitors have been developed by pharmaceutical companies, but to date no reports have been published on their effects in arthritis.

Several p38 MAPK inhibitors have reached clinical studies in humans. SB-242235 for instance has been tested for safety in healthy male volunteers and was generally well tolerated. Ex vivo, SB-242235 dose-dependently inhibited endotoxin-induced TNFα, IL-1β, IL-6 and IL-8 production, which seemed to be related to its plasma concentrations (186). RWJ-67657 was tested in a randomized, placebo-controlled human endotoxaemia trial that included 62 healthy volunteers. Treatment with RWJ-67657 dose-dependently decreased symptoms and cytokine levels, suggesting possible efficacy in the treatment of sepsis or inflammatory diseases like RA (187). Safety studies of BIRB-796, a non-aryl-pyridyl heterocyclic compound, in healthy volunteers showed asymptomatic increases in transaminases and resulted in much
more effective ex vivo inhibition neutrophil activation than TNFα production (188-190). Phase I clinical trials have also been initiated for the p38 inhibitor RO3201195 (191,192). The effects of oral administration of VX-702 and VX-745 were tested in patients with active RA. VX-745 was well tolerated and produced a significant clinical effect. However, this compound also resulted in elevation of liver transaminases and the development was suspended after pre-clinical studies indicated that the compound crosses the blood-brain barrier and could cause adverse neurological effects (193). One of the most promising and advanced p38 MAPK inhibitors presently in phase II clinical trials is SCIO-469. Another compound (SCIO-323) is currently under investigation in a phase I trial (194).

At the moment several compounds have reached Phase II and III trials and it is anticipated that p38 MAPK inhibitors could have efficacy in arthritis. However, several clinical trials have been stopped due to safety issues. This could potentially be caused by direct effects of p38 inhibition or cross-reactivities against other kinases, as almost all p38 inhibitors are ATP competitors. This warrants the development of new selective inhibitors that do not target ATP-binding and have specificity for a distinct p38 subset.

JNK inhibition
The important role of JNK in TNFα-stimulated c-jun phosphorylation and AP-1 activity, as well as in Th1 differentiation, makes it an attractive therapeutical target for many autoimmune diseases.

Table 1. Summary of p38 MAP kinase inhibitors.

<table>
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<tr>
<th>Compound</th>
<th>Company</th>
<th>Specificity</th>
<th>Reference</th>
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<tbody>
<tr>
<td>SB-203580</td>
<td>GlaxoSmithKline</td>
<td>p38α,β</td>
<td>(169)</td>
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<tr>
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<td>GlaxoSmithKline</td>
<td>p38α,β</td>
<td>(170)</td>
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<td>(171)</td>
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<tr>
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<td>RW Johnson Pharmaceutical</td>
<td>p38α,β</td>
<td>(173,187)</td>
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<tr>
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<td>Merck</td>
<td>p38α</td>
<td>(172)</td>
</tr>
<tr>
<td>pyridinyl/oxazole inhibitor</td>
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<td>(405)</td>
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<td>p38α</td>
<td>(175)</td>
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<td>RPR-238677</td>
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</table>

* Also inhibits JNK1.
Several reports about the effects of JNK inhibitors in animal models of inflammatory diseases like arthritis have been published (Table 2). The small molecule compound SP-600125, a selective inhibitor of JNK 1, 2 and 3, as well as a series of derivatives of this compound, blocks IL-1-induced phosphorylation of JNK and c-jun in cultured fibroblast-like synoviocytes from RA patients and inhibits MMP13 production. SP-600125 also blocks JNK in vivo, as subcutaneous administration inhibited JNK activation and MMP13 expression in the joints of arthritic rats, resulting in modestly decreased paw swelling and almost complete inhibition of radiographic damage (195). Additional studies indicate that SP-600125 blocks antigen-driven activation of CD8+ T cells, but in CD4+ T cells JNK inhibition selectively inhibits Th1 mediated immune responses (160,196,197), which would be beneficial in arthritis. Recently, the first selective JNK inhibitor, CC-401, was tested in a Phase I study in normal healthy volunteers (198).

Many pharmaceutical companies have put efforts in developing selective JNK inhibitors for the treatment of neurodegenerative and inflammatory diseases, but to date data in arthritis are very limited. New substituted indolizine derivatives were claimed as JNK inhibitors for the treatment of arthritis, but pharmacological data have as yet not been disclosed (199). Also, a series of pyrimidinyl-substituted benzazole-acetonitriles (200) and a large series of sulfonyl-aminoacid, sulfonamide and sulfonylhydrazides were patented as JNK2 and JNK3 inhibitors for the treatment of autoimmune diseases (201-203). It is not known whether these inhibitors are competitive for the ATP or the substrate site, or inhibit in a noncompetitive manner. Recently, a series of 4-aryl- and 4-alkynyl-isoindolones, and 4,5-pyridazinoxindoles were disclosed as JNK inhibitors (204-206). One company has prepared azoles as JNK inhibitors that

### Table 2. Summary of JNK MAP kinase inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Company</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-600125</td>
<td>Signal Pharmaceuticals/ Celgene</td>
<td>JNK2</td>
<td>(160,195-197)</td>
</tr>
<tr>
<td>indolizine derivatives</td>
<td>Aventis</td>
<td>JNK</td>
<td>(199)</td>
</tr>
<tr>
<td>pyrimidinyl-substituted benzazole-acetonitriles</td>
<td>Serono</td>
<td>JNK2,3</td>
<td>(200)</td>
</tr>
<tr>
<td>sulfonamide and sulfonyl hydrazides</td>
<td>Serono</td>
<td>JNK2,3</td>
<td>(201-203)</td>
</tr>
<tr>
<td>4-aryl- and 4-alkynyl-isoindolones</td>
<td>Hoffman-LaRoche</td>
<td>JNK</td>
<td>(204,205)</td>
</tr>
<tr>
<td>4,5-pyridazinoxindole</td>
<td>Hoffman-LaRoche</td>
<td>JNK</td>
<td>(206)</td>
</tr>
<tr>
<td>azoles</td>
<td>Takeda</td>
<td>JNK1</td>
<td>(207)</td>
</tr>
<tr>
<td>3-oximido-oxindole analogues</td>
<td>Vertex</td>
<td>JNK3</td>
<td>(208)</td>
</tr>
<tr>
<td>4-substituted isoxazole analogues</td>
<td>Vertex</td>
<td>JNK3</td>
<td>(209)</td>
</tr>
<tr>
<td>4-(4-pyrimidinyl)-5-phenylimidazole derivatives</td>
<td>Merck</td>
<td>JNK3</td>
<td>(211,212)</td>
</tr>
<tr>
<td>CNI-1493*</td>
<td>Picower Institute</td>
<td>JNK1</td>
<td>(178,179,181,182)</td>
</tr>
<tr>
<td>Compound 11</td>
<td>Eisai</td>
<td>JNK3</td>
<td>(210)</td>
</tr>
<tr>
<td>CEP-1347 (KT7515)</td>
<td>Cephalon Inc.</td>
<td>MLK1,2,3</td>
<td>(213)</td>
</tr>
</tbody>
</table>

* Also inhibits p38.
have been demonstrated to efficiently inhibit TNFα production in vitro (207). Lastly, a series of JNK3 inhibitors was patented for the treatment of stroke and neurodegenerative diseases (208-212). Another way of targeting JNK is by inhibiting its upstream activators. CEP-1347 (KT7515), a small molecule that inhibits mixed lineage kinases, is a potent anti-inflammatory compound in neurodegenerative diseases, but has not been tested in arthritis yet (213).

Most of the described kinase inhibitors either interfere with phosphorylation or bind competitively in the ATP binding site and, therefore, are bound to be less specific, because they also have (limited) effects on other kinases. With increasing knowledge about the cellular functions of JNK1, JNK2 and JNK3, the next step will probably be dominant-negative forms or (cell type specific) membrane permeable peptide inhibitors of the individual JNK proteins.

**ERK inhibition**

Compared to the development of p38 and JNK inhibitors, the development of ERK1/2 inhibitors has lagged behind. Because of the importance in osteoclast differentiation ERK inhibition research has focused on the effects on bone erosion and joint destruction rather than on the anti-inflammatory effects. Many companies have developed MEK1/2 inhibitors, some of which are commercially available (Table 3).

PD098059, an inhibitor of MEK1/2 and thereby a blocker of ERK activation (214,215), reduces proliferative responses, c-Jun phosphorylation and MMP-1 gene expression in IL-1 stimulated fibroblast-like synoviocytes (195,216). U0126, an inhibitor of AP-1 transactivation in a cell-based reporter assay, also selectively inhibits endogenous promoters containing AP-1 response elements. The effects of U0126 also result from direct inhibition of the MAPK kinase family members, MEK-1 and MEK-2 (217). Both PD098059 and U0126 do not appear to compete with ATP and bind to similar sites on MEK as they compete with each other for binding. The second generation, more potent MEK-1/2 inhibitor PD184352 has enhanced bioavailability and also appears to act via an allosteric mechanism. PD184352 does not inhibit JNK, p38 or Akt, and is currently tested in Phase I oncology trials (218,219). Recently,

### Table 3. Summary of ERK/MEK MAP kinase inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Company</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD098059</td>
<td>Warner-Lambert</td>
<td>MEK1/2</td>
<td>(214,215)</td>
</tr>
<tr>
<td>U0126</td>
<td>Merck</td>
<td>MEK1/2</td>
<td>(217)</td>
</tr>
<tr>
<td>PD184352</td>
<td>Warner-Lambert</td>
<td>MEK1/2</td>
<td>(218,219,407)</td>
</tr>
<tr>
<td>3-cyano-4-(phenoxyanilino)quinolines</td>
<td>Wyeth-Ayerst</td>
<td>MEK1/2</td>
<td>(220)</td>
</tr>
<tr>
<td>RO092210</td>
<td>Roche</td>
<td>MEK</td>
<td>(221)</td>
</tr>
<tr>
<td>L783277</td>
<td>Merck</td>
<td>MEK</td>
<td>(222)</td>
</tr>
<tr>
<td>LLZ16402</td>
<td>Ajinomoto Co.</td>
<td>MEK</td>
<td>(223)</td>
</tr>
<tr>
<td>GP790</td>
<td>Metabasis Therapeutics Inc.</td>
<td>adenosine kinase</td>
<td>(225)</td>
</tr>
<tr>
<td>oxindoles</td>
<td>GlaxoWellcome/Merck</td>
<td>Raf-1</td>
<td>(228)</td>
</tr>
<tr>
<td>BAY43-9006</td>
<td>Bayer</td>
<td>Raf-1</td>
<td>(229,230)</td>
</tr>
</tbody>
</table>
a series of 3-cyano-4-(phenoxyanilino)quinolines were identified as novel MEK inhibitors, but the mechanism of action has not been disclosed (220). Several other MEK inhibitors, like RO092210 and L783277, have been isolated from microbial extracts that are competitive with ATP and also block MEKs 4, 6 and 7 (221,222). Furthermore, LLZ16402, identified as an inhibitor of phorbol-ester-induced AP-1 transcription, might also be a MEK inhibitor (223). Although the presence of MEK inhibitors in micro-organisms is interesting and could lead to the development of promising new compounds, the relative selectivity of these compounds among MEK family members remains unclear and needs further evaluation. In contrast to the MEK inhibitors, only few selective ERK1/2 inhibitors have been reported. Of note, one interesting study revealed that substitution of one amino acid makes ERK2 susceptible to pyridinyl imidazole inhibitors of p38 MAP kinase and identified the adenosine kinase inhibitor 5-iodotubercidin as a potent but not very specific ERK2 inhibitor (224). A structurally related but more selective compound, GP790, was demonstrated to have beneficial anti-inflammatory effects in the carrageenan paw edema model of inflammation (225). To block ERK DNA binding activity, ERK antisense and scrambled oligonucleotides were developed (226), which could be used for local application in vivo, such as intra-articular injection.

The MEK/ERK inhibitors U0126, PD098059 and ERK1/2 antisense phosphorothioate oligonucleotides all down-regulate TNFα stimulated expression of MMP13 in articular chondrocytes and could, therefore, be beneficial in the prevention of cartilage destruction (227).

Considerable effort has also been put in developing Raf-1 inhibitors, mainly in the search for new anti-cancer drugs. Potent Raf-1 inhibitors from a series of oxindoles were reported, as well as a Raf-1 inhibitor that competes with ATP (228). To date, the most advanced Raf-1 inhibitor is BAY43-9006, which has demonstrated efficacy in cell and animal assays, and is now in phase I trials in cancer (229,230). At present, no MEK/ERK inhibitors have been tested in animal models of RA. However, the effects of PD098306 were recently evaluated in a rabbit model of osteoarthritis (OA). Oral administration of the drug partially decreased the development of some of the structural changes in experimental OA. This effect was associated with a reduction in the level of phospho-ERK-1/2 in OA chondrocytes (231). Whether ERK inhibition also has beneficial effects in (models of) RA still needs to be tested.

The explosion in design of p38, JNK and ERK inhibitors, together with the important role of MAPKs in inflammatory responses, stresses the idea that interfering with MAPK signal transduction could be a promising new tool in the treatment of autoimmune diseases like RA. However, it remains to be shown whether clinical efficacy can be achieved with acceptable safety.
PI3K/AKT PATHWAY

The phosphoinositide 3-kinase (PI3K) pathway represents a major signaling pathway involved in a variety of cellular responses, including cell growth, differentiation, survival, proliferation and migration (Figure 3). Although the PI3K family contains various isoforms with diverse biological functions, the class Ia enzymes are primarily responsible for the response to growth factors. PI3K is a heterodimer, consisting of a regulatory subunit (p85α, β or p55γ) and a catalytic subunit (p110α, β, γ, δ). PI3K activity is stimulated by recruitment to cellular membranes, either as a result of regulatory subunit binding to phosphotyrosine kinase (PTK) substrates, or in some cases, by recruitment to activated Ras GTPase. Activation of PI3K results in phosphorylation of phosphatidylinositol, which acts as a lipid second messenger and binds to various signaling proteins, including protein serine-threonine kinases, protein tyrosine kinases and exchange factors that regulate GTP-binding proteins. This results in membrane localization and activation of the target protein. One of the most prominent signaling proteins in this cascade is Akt (also known as protein kinase B), which is phosphorylated.

Figure 3. Schematic representation of the PI3K/Akt signal transduction pathway.
Phosphoinositide 3-kinase (PI3K)/Akt can be activated by a multitude of different stimuli, like growth factors, integrins and cytokines. Signaling pathways downstream of PI3K/Akt affect cell growth, cell survival, and cell movement. PI3K is brought to the membrane and activated by directly binding to phosphotyrosine residues of receptors or adaptors. The lipid product of PI3K recruits a subset of signaling molecules, including protein serine-threonine kinases (Akt and PDK) and exchange factors for GTP-binding proteins like Rac and ARF6, to the membrane, where they are activated. Ultimately, these proteins initiate complex sets of events that control protein synthesis, actin polymerization, cell survival and cell cycle entry. Of note, some of these events are mediated via the effects of PI3K/Akt on other signal transduction pathways like NF-κB and ERK MAPK (see Figures 1 and 2).
by PDK1. Activated Akt phosphorylates proteins that affect cell growth, cell cycle entry, and cell survival (reviewed in (232)). Forkhead box class O (FOXO) factors become inactivated by Akt-mediated phosphorylation, via cytosolic retention and prevention of gene transcription (233). Similarly, Akt-mediated phosphorylation of pro-apoptotic proteins like Bad and down-regulated expression of FasL prevents apoptosis and results in cell survival (234). Other proteins that are activated by PI3K include GDP-GTP exchange factors for Rac and ARF6 that play a major role in actin cytoskeleton remodeling in response to chemotactic agents. Termination of PI3K signaling by degradation of phosphorylated phosphatidylinositol can be achieved by phosphatases like the Src-homology 2 (SH-2)-domain-containing inositol polyphosphate phosphatases SHIP1 and SHIP2 and the phosphatase and tensin homologue PTEN (235).

In the immune system distinct members of the PI3K family with different biological functions are activated, according to the cell type and receptor. In B cells PI3K is involved in B cell development and in B cell receptor and CD19 signaling, whereas in T cells PI3K is required for normal T cell development, differentiation of naïve T cells into effector, regulatory and memory T cells, T cell receptor signaling and CD28 signaling (reviewed in (236)). Unrestrained PI3K signaling may contribute to autoimmunity as observed in Pten+/- mice (237). In addition, mice with a Cre-mediated deletion of PTEN restricted to T cells show impaired thymic negative selection, leading to lymphoproliferation and autoimmunity (238). Other more widely distributed receptors like IL-1 receptors, Toll-like receptors and members of the TNF-R family, including CD40, also activate PI3K in many cell types like macrophages and DC. A collection of studies demonstrate the diversity of roles played by PI3Ks in the innate immune system, including phagosome formation, chemoattractant-induced neutrophil migration and oxidative burst (239), and normal mast cell formation and distribution (175) (240). These effects are at least partly mediated via interaction of PI3K with other signal transduction pathways like NF-κB, NFAT and the JAK/STAT pathway (reviewed in (241)).

In macrophages and DC from p85-/- mice, lack of PI3K activity enhanced p38 MAPK activity via an unknown mechanism, resulting in increased IL-12 production and Th1 cell differentiation. In vitro studies demonstrated that PI3K inhibition in wild-type DC by wortmannin also resulted in increased IL-12 synthesis (242). Consistent with this, in monocytes PI3K has a suppressive role in TLR-induced MAPK activation, resulting in decreased TNFα production (243). Thus, PI3K-mediated negative feedback seems to be important in preventing excessive innate immune responses, suggesting that PI3K is one of the key regulators in the Th1 versus Th2 balance, predominantly through control of IL-12 production. In general, activation of PI3K seems to positively regulate cell growth and survival, but can negatively regulate other cellular responses like p38 MAPK mediated IL-12 production by DC.

The role of PI3K/Akt in RA

Immunohistochemistry revealed that Akt-1 is expressed at higher levels in RA synovium compared to OA. Furthermore, RA fibroblast-like synoviocytes were more susceptible to TNFα
induced Akt-1 activation, which could be suppressed by inhibition of PI3K by wortmannin (244). PI3K appears to be especially important in preventing TNFα-induced apoptosis as transfection of fibroblast-like synoviocytes with an adenoviral construct expressing dominant-negative Akt, overexpression of PTEN, or treatment with wortmannin renders these cells sensitive to apoptosis. This indicates that phosphorylated Akt acts as a survival signal in RA fibroblast-like synoviocytes and contributes to the stimulatory effect of TNFα on these cells by inhibiting apoptosis. Together with findings that RA synovium lacks PTEN expression at sites of invasive fibroblast-like synoviocyte growth (245), this might explain in part the impaired apoptosis associated with the proliferating synovium in RA.

In macrophages the PI3K/Akt pathway is also important, as Akt-1 is constitutively activated in macrophages in vitro. This appears to be crucial for cell survival because suppression of Akt-1 by a PI3K inhibitor or dominant-negative Akt-1 results in apoptosis, even in the absence of an additional pro-apoptotic signal (246). Macrophages isolated from synovial fluid of RA patients also go into apoptosis following inhibition of the PI3K pathway (247). Intriguingly, suppression of PI3K in normal peripheral blood derived macrophages with the inhibitors LY294002 or wortmannin resulted in enhanced production of the pro-inflammatory cytokine TNFα and inhibited production of the anti-inflammatory cytokine IL-10 after CD40 ligation, suggesting a direct negative regulation of TNFα and a positive regulation of IL-10 by PI3K (248). In RA synovial tissue-derived mononuclear cells the spontaneous production of IL-10 was inhibited by LY294002, but not by wortmannin (249). This differential response to wortmannin between macrophages and synovial mononuclear cells could be due to a potency effect or differential utilization of PI3K isoforms.

Taken together, these findings suggest that in RA macrophages and fibroblast-like synoviocytes the anti-apoptotic effects of the PI3K/Akt-1 pathway may account in part for the increased numbers and proliferation of these cells. However, in macrophages and synovial mononuclear cells PI3K also seems to have an anti-inflammatory role. Given the differential effects of PI3K activation and inhibition on immune cells, it is as yet difficult to predict the consequences of PI3K/Akt inhibition in RA. Therefore, it is important to increase our understanding of this pathway and the role of its isoforms in different cell types, both in normal biology and in pathology.

PI3K/Akt inhibition

Currently, several pharmaceutical companies are developing PI3K isoform-selective inhibitors that would allow for a more sophisticated research approach in dissecting the PI3K pathway than the currently available non-isoform specific inhibitors LY294002 and wortmannin. Recently, several p110-isoform-specific inhibitors were developed, which have already resulted in a greater understanding of the role of this PI3K isoform in certain cell types and conditions (250-253). Several imidazopyridine derivatives have been patented that are claimed to be p110α specific, although no isoform selectivity data are provided (254). Also, a series of
morpholino-substituted compounds that are closely related to LY294002, but with isoform selectivity against p110α and p110β, were recently disclosed (255). p110δ specific inhibitors have also been generated, of which IC87114 is the first isoform-specific PI3K inhibitor characterized biologically (251,252). IC87114 has an IC50 of 0.5uM and exhibits >50 fold selectivity over other class I PI3K isoforms. Finally, some natural compounds like the methylxanthines caffeine and theophylline, have been reported to be selective for p110δ isoforms over p110α and p110β isoforms, although their activity is fairly low (256).

The therapeutic potential of isoform-selective PI3K inhibitors in general is still untapped. Whether PI3K inhibition will have a role in the future treatment of RA remains to be seen and requires more understanding of the role of this pathway and its isoforms in the pathogenesis of RA.

**JAK/STAT PATHWAY**

Stimulation of a cell by certain cytokines and growth factor activates a distinct pair of the 4 known cytoplasmic janus tyrosine kinases (JAKs) that associate with the type I/II cytokine receptor. Activated JAKs phosphorylate tyrosine residues on these receptors and thereby recruit latent cytoplasmic transcription factors named signal transducers and activators of transcription (STATs). STATS are subsequently activated by phosphorylation that results in release from the receptor complex, allowing dimerization and translocation to the nucleus. Here, they can bind to specific binding sites on DNA and activate transcription. DNA binding of STATs can give rise to transcription of many inflammatory effector genes, including immunoglobulin receptors and cell surface proteins like ICAM-1. STATS are also implicated in regulation of cell growth, differentiation and survival, defining dual roles for STATS in both normal homeostasis and inflammatory responses (Figure 4).

At present, 7 mammalian STATs have been identified: STAT 1-STAT 6. STAT 5A and STAT 5B are 91% identical and can be activated by the same cytokines. However, they are encoded by different genes and have different biological functions (reviewed in (257,258)).

STATs are able to bind two types of DNA motifs, ISREs (IFN stimulated response elements) and GAS elements (γ activated sequence), that contribute to the specificity of cytokine-induced gene expression (259). In the tightly regulated activation process of these transcription factors, tyrosine phosphorylation is an essential step. The JAK family comprises 4 protein tyrosine kinases: JAK1, JAK2, JAK3 and Tyk2. All type I and II cytokine receptors signal via various JAKs (JAK1, JAK2 and Tyk2), whereas JAK3 is only involved in cytokine signaling via receptors that contain the common cytokine γ chain (IL-2, IL-4, IL-7, IL-9 and IL-15).

Some aspects of JAK/STAT signaling appear to be conserved with that of NF-κB, as a family of endogenous inhibitors of STATs has been described (260-262). The cytokine-inducible SH2 containing (CIS) family of proteins (also referred to as the suppressor of cytokine signaling
Signal Transduction Pathways and Transcription Factors as Therapeutic Targets

(SOCS) family) has been implicated in the regulation of JAK/STAT signal transduction. They appear to be induced after stimulation with different cytokines, and at least three of them (CIS1, CIS3/SOCS3, and JAB/SOCS1) negatively regulate signal transduction. CIS1 inhibits STAT5 activation by binding to cytokine receptors that recruit STAT5, but JAB/SOCS-1 and CIS3/SOCS-3 directly bind to the kinase domain of JAKs, thereby inhibiting tyrosine-kinase activity. Therefore, these CIS family members seem to function in a classical negative feedback loop of cytokine signaling (263).

The role of JAK/STAT in RA
Altered JAK/STAT signaling appears to play an important role in RA as immunohistochemistry of the inflamed synovium revealed elevated expression of STAT1, STAT3, STAT4 and STAT 6 (264-266). Using DNA microarray technology, van der Pouw Kraan et al. (267) demonstrated that STAT1 target genes are expressed in RA synovium, suggesting STATs in RA synovium are fully functional and play an active role in the disease process.

Also, constitutive STAT3 DNA binding activity has been described both in synovial fluid mononuclear cells and soluble factors in RA synovial fluid have been demonstrated to induce
activation of STAT3 in monocytes from healthy controls (268,269). IL-6 has been reported to be the major STAT3 activating factor in synovial fluid, and this cytokine was also responsible for the activation of STAT3 in synovial fluid neutrophils (269,270). IL-6 is also a good candidate for the activation of STAT3 tyrosine phosphorylation in vivo (271) as it is highly expressed in RA synovium and is the major STAT3 activating factor in synovial fluid. From a clinical point of view STAT3 is also interesting as the gene encoding the acute phase C-reactive protein (CRP) is a STAT3 target gene (272).

Although IFNγ is still considered the strongest activator of STAT1, other cytokines like IL-6, IL-10 and IFNα/β may also contribute to STAT1 activation in the synovium. Distinct, but not fully understood, mechanisms of synovial STAT1 activation may be via the B cell antigen receptor or chemokine receptor (CCR) 5 (265). Apart from signaling that leads to inflammatory responses, STAT proteins may also play an important role in cell proliferation and lymphocyte differentiation. STAT1 seems to be important in promoting caspase-1-mediated apoptosis in several cell types like lymphocytes and fibroblast-like synoviocytes (273-275). STAT1 possibly exerts this function in controlling cell proliferation by the interaction with p53 to enhance DNA damage-induced apoptosis (276).

Studies in animal models of arthritis have shown that STAT1 deficiency results in exacerbation of the disease (277). Taken together, these findings suggest both a protective and a pathogenic role for STAT1 in synovitis, probably depending on the cell types involved and the stage of the disease, consistent with the dual effects of IFNγ on synovitis that can be observed.

STAT3 likely has a causal role in experimental arthritis as treatment with a recombinant adenovirus carrying dominant-negative STAT3 or its endogenous cytokine signaling repressor CIS3/SOCS3 blocked IL-6-gp130-JAK-STAT signaling and drastically reduced the severity of arthritis in CIA and AA, together with reduced levels of IL-6 and TNFα in the joints (271). Using conditional gene targeting to generate mice lacking Socs3 in the liver or in macrophages, Croker et al. show that Socs3 deficiency resulted in prolonged activation of STAT1 and STAT3 after IL-6 stimulation, but normal activation of STAT1 after stimulation with IFNγ (278).

Further evidence comes from a study describing that an IL-6 receptor knock in mutation caused hyperactivation of STAT3 resulting in spontaneous development of inflammatory arthritis in mice (279). One of the mechanisms involved is STAT3-mediated suppression of fibroblast-like synoviocyte apoptosis (275). STAT3 also promotes T cell survival and antibody production by plasma cells, leading to sustained inflammation (280). However, STAT3 also has anti-inflammatory effects, predominantly in myeloid cells like macrophages and DC, in which it plays a critical role in suppressing pro-inflammatory genes and production of chemokines and cytokines (e.g. TNFα) (281,282). All together STAT3 could be an attractive therapeutic target in RA. However, the dual effects of STAT3 in arthritis necessitate a selectively targeted approach of inhibition, sparing the anti-inflammatory effects it has in macrophages.
It has been reported that STAT4 and STAT6 are important for Th1/2 differentiation of T helper cells. STAT4-/- mice have defective cell mediated immune responses and augmented Th2 development (283,284), implicating an important role for STAT4 in Th1 differentiation. Furthermore, these mice also show less inflammation in the proteoglycan-induced arthritis model (285). Taken together, this suggests that targeting STAT4 in Th1 mediated diseases like RA might be beneficial. In contrast to STAT4 deficiency, STAT6-/- mice have defective Th2 development, more severe arthritis, and attenuated allergic and asthmatic disease (286-288), disqualifying this protein as a potential therapeutic target in RA unless levels can be selectively increased.

**JAK/STAT inhibition**

The dual nature of the STAT proteins makes them difficult to target, which is further complicated by the fact that, unlike the JAKs, they lack enzymatic activity. A possible approach would be to target their DNA binding capacity that depends on phosphotyrosine-SH2 interaction (289). Another difficulty in targeting STAT proteins is that the inhibition should be cell type-specific, which can be hard to achieve. At present small molecule inhibitors of STATs have not been successfully developed. A more promising strategy is to induce endogenous STAT inhibitors, the SOCS, or to introduce recombinant forms of these proteins (290). Another strategy would be to design selective inhibitors of JAK kinases. Relatively selective inhibitors of JAK kinases already exist (291-294).

Preliminary studies with the JAK2 inhibitor AG490 suggested efficacy in the CIA model (271,295). There are indications that JAK1 inhibitors would be even more effective in inflammatory diseases like RA, because gp130 mainly uses JAK1 and in contrast to JAK2, JAK1 is not essential for hematopoiesis. Specific inhibition of JAK3 in articular chondrocytes blocked oncostatin M (IL-6 superfamily) -induced STAT1 tyrosine phosphorylation, DNA binding and MMP expression, whereas JAK2 inhibition by AG490 did not have these effects in chondrocytes (296,297). The reduction in MMP expression after JAK3 inhibition could result in inhibition of matrix degradation and bone erosion, which would be beneficial in destructive RA. JAK3 is preferentially expressed in hematopoietic cells and is upregulated upon cell differentiation and activation. It is activated through the γ chain and plays a critical role in T cell development and function (298,299). Inhibition of JAK3 by the selective inhibitor PNU156804 resulted in prolonged allograft survival in a rat heart transplantation model. This specific JAK3 inhibitor acted synergistically with cyclosporine. Combination therapies of this inhibitor with lower dosages of more non-specific immunotherapeutic drugs may reduce the myelosuppressive and toxic effects of these drugs (291). This may also hold true for the combination of a selective JAK inhibitor with widely used DMARDs like methotrexate. Targeting proteasome degradation, phosphorylation, cAMP levels, and cell differentiation can probably also affect the levels of the endogenous inhibitors of JAKs or STATs and partially induce inhibition, but that approach is less elegant and less controlled than the methods described above.
Glucocorticoids have differential effects on JAK/STAT signal transduction, resulting in cell type and cytokine-specific outcomes and cross-talk between JAK/STAT and glucocorticoid pathways. Glucocorticoids are thought to control the signaling amplitude of pro-inflammatory cytokines like IFN\(_\gamma\), IL-12 and IL-2, by regulating expression or activation of STAT1, STAT4 and STAT5 respectively (300-302).

A better understanding of the role of the JAK/STAT pathway in arthritis is needed to rationally design highly specific inhibitors of this signaling cascade for therapeutic use in RA.

**P53 SIGNALING**

The tumor suppressor transcription factor p53 is important in DNA repair, thereby controlling apoptosis and cell replication. p53 consists of a transactivation region that stimulates transcription of a number of genes involved in tumor suppression, and a transrepression region that suppresses (proto)oncogenes (reviewed in(303)).

Induction of p53 occurs when DNA is damaged after ultraviolet irradiation, exposure to oxygen free radicals, oncogenes such as c-myc, or inflammatory mediators (304-307). p53 induces cell cycle arrest and may result in either DNA repair or apoptosis. Mutations in the p53 gene may have dramatic effects, especially when this results in a dominant-negative protein that blocks normal p53 function, leading to resistance to apoptosis and uncontrolled cell division.

The role of p53 in RA

Several studies have shown that p53 expression is upregulated in the intimal lining layer of RA synovial tissue and in experimental models of arthritis (304,308-311). p53 down-regulates human MMP1 gene expression (312) and consequently mutations may lead to increased MMP expression. Of importance, dominant negative p53 mutations, associated with oxidative damage have been detected in rheumatoid fibroblast-like synoviocytes (313-315). These cells may have a “transformed” phenotype and are characterized by invasive, anchorage independent growth, increased proliferation, resistance to apoptosis, as well as spontaneous expression of proto-oncogenes and expression of anti-apoptotic proteins like Bcl-2, Bcl-x\(_L\) and sentrin-1 (316,317).

Further evidence for the relevance of p53 in fibroblast-like synoviocytes in vivo comes from studies showing that DBA/1 p53\(^{-/-}\) mice developed more severe experimental arthritis with increased IL-6 expression than wild-type mice (318). Inhibition of p53 was associated with increased cellularity and cartilage invasion in a mouse model when RA fibroblast-like synoviocytes were implanted into severe combined immunodeficiency (SCID) mice (319). Wild-type p53 interacts with multiple signal transduction pathways, for instance by blocking proliferation of cells that have been transformed by Ras mutations (320), serving as a substrate for JNK
(321), suppressing IL-6 promoter activity (322) and inhibition of IL-6 signal transduction by inducing STAT-masking activity (323).

**p53 targeted therapies**

It has been suggested that p53 might have therapeutic potential in RA. This is largely based on a study that demonstrated that intra-articular injection of an adenoviral vector expressing wild-type p53 induced synovial apoptosis and reduced inflammation, without affecting cartilage metabolism (324). Recently, similar data were obtained using peptides that bound to a defined site on p53 and stabilized it against denaturation *in vitro*. Although this did not elicit a full biological response, there was p53-dependent induction of expression of p53 target genes, accompanied by p53-dependent partial restoration of apoptosis (325). It should be noted that these effects and in particular the clinical relevance need to be confirmed. In addition, it is unclear how much functional p53 is present in the joints of RA patients.

Some of the drugs that are currently used in the treatment of RA like methotrexate and sulfasalazine have effects that might be mediated in part by induction of apoptosis; this is mainly mediated via caspases rather than via p53 (326-328).

Taken together, with increasing understanding of the role of impaired apoptosis in RA it should be possible to rationally design cell type specific, small molecules that induce apoptosis in the inflamed synovium. Whether this could be achieved by activating p53 function remains to be shown (329).

**REACTIVE OXYGEN SPECIES (ROS) AND OXIDATIVE STRESS**

Over the past several years the concept that reactive oxygen species (ROS) have purposeful roles as regulated second messengers in cellular signaling pathways has gained significant recognition. It is now generally accepted that ROS act as vital mediators of both physiological and inflammatory signal transduction pathways (330-332). The evidence supporting this concept is based largely on the following criteria: 1) growth factors and cytokines are capable of generating ROS in a number of different cell types, 2) antioxidants and inhibitors of ROS-generating enzymatic systems block specific growth factor- and/or cytokine-activated signaling events or physiological effects, and 3) exogenous addition of oxidants can mimic activation of signaling cascades by the same cytokines and/or growth factors. Additionally, extracellular release of ROS at inflammatory sites, via the superoxide burst of activated neutrophils and monocytes, cationic catalysts released by damaged or necrotic cells, and ischemia/reperfusion, can modify cell function either through oxidation of cell surface molecules or permeating neighboring cells to influence intracellular signaling pathways (333,334).
Reduction-oxidation (redox) reactions that generate ROS have been identified as important chemical mediators in the regulation of signal transduction processes involved in cell growth and differentiation (reviewed in (335,336)). Moreover, the rapid kinetics of activation and inactivation, and the intrinsic characteristics of ROS make them ideal intracellular messengers for fine-tuning transient activation of intracellular signaling pathways.

Although a number of growth factors and cytokines that bind to different classes of receptors have been demonstrated to raise intracellular ROS, little is known about the molecular mechanisms regulating intracellular ROS generation. ROS generation in response to cytokine receptor stimulation has been reported for TNFα, IL-1, and IFNγ, generally within the context of apoptotic pathways induced by these agonists. However, there is little consensus on the specific ROS species produced, their enzymatic source, or where they are generated within the cell. Potential sources include 5-lipoxygenase, xanthine oxidase (XO), COX, nitric oxide synthases (NOS), NADPH oxidases, and mitochondrial respiration (337). In many cell types, extracellular stimuli or stress can induce mitochondrial permeabilization, also leading to elevation of intracellular ROS. In fibroblasts, many extracellular stimuli generate ROS via a signaling pathway requiring NADPH oxidase assembly by signaling from the small GTPase Ras to Rac GTPases (338,339). In neutrophils, activation of Rac is a requisite step in the generation of ROS (340,341). In T cells, TCR stimulation also generates ROS by a Ras-dependent mechanism, although here ROS production does not involve NADPH, and the GTPase Ral links Ras to ROS production. Also in T cells, a third GTPase, Rap1 has recently been shown to play a novel role in attenuating Ras-dependent ROS production (342).

ROS in RA

ROS are thought to play several distinct roles in the pathophysiology of RA (343). First, ROS produced by activated macrophages and neutrophils, ischemia/reperfusion- compromised oxygen radical tension in the inflamed joint, and generation of hydroxyl radicals by Fe2+ released from dying cells (344,345). These sources of ROS could lead to oxidative damage of surrounding cells and cartilage (346). Additionally, ROS-dependent activation of T lymphocytes by synovial neutrophils has been reported (347). Second, transient production of ROS by TNFα and other inflammatory stimuli, plays a key role in NF-κB-dependent transcription of inflammatory cytokines and MMPs, perpetuating inflammation and joint destruction (348,349). Activation of this transcriptional pathway will also mediate transcription of proteins, such as FasL, FLIP, and Bcl-2 family members, involved in determining survival/apoptotic cell fate decisions. Third, chronic oxidative stress, either from external sources or intracellular signaling pathways generating ROS, may substantially alter cell function (350-352). In RA synovial fluid T cells, chronic oxidative stress leads to misfolding of key intracellular signaling proteins required for TCR-dependent proliferative responses, while simultaneously enhancing NF-κB-dependent transcription of TNFα and IL-1 receptors (353). Recent evidence suggests that TNFα stimulation may drive chronic oxidative stress in these T cells (354,355).
Constitutive Ras activation and blocked Rap1 activation in RA synovial T lymphocytes are likely responsible for the observed spontaneous ROS production (342). Lastly, chronic exposure of fibroblast-like synoviocytes to ROS has been proposed to mediate genotoxic damage of p53 and other tumor suppressors, leading to autonomous disease progression in RA (reviewed in (309)).

Although ROS have been demonstrated to be involved in many signal transduction pathways their specific molecular targets are not yet precisely defined. Current concepts of ROS signaling can be divided into two general mechanisms of action. The first is oxidative alterations of protein structure (or dimerization) and protein function by modifying critical amino acid residues, often cysteines. Such alterations may alter the activity of an enzyme if the critical amino acid residue is located within its catalytic domain (356) or the ability of a transcription factor to bind DNA if it is located within its DNA-binding motif (357). The second mechanism of action relates to the intracellular redox state. In comparison with the extracellular environment, the cytosol is normally maintained under strong reducing conditions, because it is essential that signaling systems are kept in a reduced state for optimal functioning. This is mainly accomplished by the redox-buffering capacity of intracellular thiols, primarily glutathione (GSH) and thioredoxin (TRX), but many antioxidative defense mechanisms contribute to this process. In addition to antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, a broad range of non-enzymatic scavengers are known. These buffering systems are indispensable for keeping signaling molecules in a reduced state and also scavenge intracellular ROS generated in the signaling process, thereby suppressing downstream targets of ROS. The cumulative effect of oxidative stress is thus very complex and modifies multitude levels of effectors in the signaling pathways from receptor to nucleus (Table 4).

Table 4. Signaling molecules regulated by redox balance.

<table>
<thead>
<tr>
<th>Signaling molecule</th>
</tr>
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<tbody>
<tr>
<td>1. a. <strong>Protein Tyrosine Kinases</strong></td>
</tr>
<tr>
<td>EGF receptor, insulin receptor, PDGF receptor, Src, Lck, Fyn, ZAP-70, Syk, Lyn, Fgr, Hck, Btk, Ltk</td>
</tr>
<tr>
<td>b. <strong>Protein Tyrosine Phosphatases</strong></td>
</tr>
<tr>
<td><strong>Lipid Signalling</strong></td>
</tr>
<tr>
<td>PLC, PLD, PLA₂, PI3 kinase</td>
</tr>
<tr>
<td><strong>Ca²⁺ Signalling</strong></td>
</tr>
<tr>
<td>Ins(1,4,5) receptor, Ca²⁺ATPase</td>
</tr>
<tr>
<td><strong>Small G proteins</strong></td>
</tr>
<tr>
<td>Ras, Rap1</td>
</tr>
<tr>
<td><strong>Protein Serine/Threonine Kinases</strong></td>
</tr>
<tr>
<td>JNK, p38, Akt, PKC</td>
</tr>
<tr>
<td><strong>Protein Serine/Threonine Phosphatases</strong></td>
</tr>
<tr>
<td>PP1, PP2A, calcineurin</td>
</tr>
<tr>
<td><strong>Transcription factors</strong></td>
</tr>
<tr>
<td>AP-1 (c-fos, c-jun), NF-κB, p53</td>
</tr>
</tbody>
</table>
ROS and Protein Tyrosine kinases (PTK)

Although the binding of a growth factor to its receptor and the subsequent activation of its receptor tyrosine kinase (RTK) domain or associated kinases initiates signal transduction pathways upstream from intracellularly generated ROS, receptor kinases and phosphatases themselves may also be targets of oxidative stress. Oxidants such as H₂O₂ have been shown to induce phosphorylation of the epidermal growth factor (EGF) receptor and the platelet derived growth factor (PDGF) (358,359). Although oxidative stress apparently stimulates many PTKs in cells, there is no evidence so far that PTKs are direct targets of H₂O₂. A study by Knebel et al. (360) suggests that the mechanism of these effects may be related to ROS-mediated inhibition of the dephosphorylation of RTKs by inactivation of protein tyrosine phosphatases. Not only RTKs, but also intracellular PTKs such as Src family members are activated by free radicals. H₂O₂ has been shown to induce tyrosine phosphorylation and the activation of many PTKs including Lck (361,362), Fyn (363) and ζ-associated polypeptide of 70kDa (ZAP-70) (364) in T cells. ROS also interact with the PI3K/Akt pathway, as activated RTKs can stimulate PI3-kinase, which in turn activates several protein kinases such as Akt and PKCs (365).

In all, the reports on the activation of RTKs and PTKs relate primarily to the effects of exogenously added oxidants. The significance of such actions in specific growth factor- or cytokine-mediated signaling is less clear. However, this does not exclude the possibility that certain physiological stimuli, or oxidative stress, may lead to ROS-dependent activation of cellular PTKs.

ROS and MAPK

Because the MAPK pathways mediate both mitogen- and stress-activated signals, there has been significant interest in the redox regulation of these pathways. Most reports have concentrated on ROS-dependent JNK and p38 responses, but the mitogenic ERK MAPK, and regulatory proteins for all three of these MAPKs, have also been implicated as potential targets of ROS (reviewed in (366)). Especially in T cells, there is abundant evidence that ROS induce activation of p38, JNK and ERK (367,368).

Redox regulation of Transcription Factors

Oxidative stress induces the expression of many genes such as c-fos, c-jun, c-myc and a gene for haeme oxygenase. This expression is mediated by transcription factors in response to the activation of upstream cellular signaling pathways. Indeed, treatment of cells with H₂O₂ induces the activation of the transcription factors AP-1 and NF-κB in some cell types (330). However, it should be noted that oxidative stress can also interfere with the activity of many of these transcription factors.
NF-κB

NF-κB has long been considered oxidant responsive (369,370). Free radicals activate NF-κB in response to the activation of upstream cellular signaling pathways. However, for NF-κB to be functional, the DNA binding region needs to be kept in a reduced state. Additionally, a critical step in NF-κB activation is the phosphorylation of IκB. IKK -dependent phosphorylation of IκB is also sensitive to oxidative stress, down-modulating NF-κB activation. This redox-regulated effect occurs downstream from IKK, at the level of ubiquitination and/or degradation of IκB (371). ROS can also influence NF-κB DNA binding as TNFα induces a delayed ROS-dependent signaling pathway that is required for NF-κB transcriptional activation and is separable from that required for its nuclear translocation (348,349). In contrast, exposure to oxidative stress in certain cell types induced reduction of TNF-induced NF-κB activation by inhibition of IKK (372). Recent reviews (373,374) on this subject have, therefore, emphasized the dual regulation of NF-κB by oxidative stress and the importance of the recognition that a redox-dependent activation of NF-κB is cell type- and stimulus- specific, a caution on concluding that ROS are solely activators of NF-κB.

AP-1

The transcription factor AP-1 also appears to be activated by antioxidants (375,376). In vitro experiments suggest that a single cysteine residue in the highly conserved tri-amino acid sequence Lys-Cys-Arg of the DNA binding domain of Fos and Jun proteins confers redox sensitivity to AP-1 (357). The reduced state of this cysteine residue is essential for its DNA-binding and transformation activity. Thus, oxidative stress is likely to directly interfere with AP-1-dependent gene transcription.

p53

Complex cysteine residue interactions make p53 highly sensitive to redox regulation (377). p53 binds to Zn\(^{2+}\) through these cysteine residues. The ability of p53 to regulate cell cycle arrest or apoptosis depends upon the activity of this site. The activity of p53 is modulated by copper through a redox mechanism, and treatment with anti-oxidants disrupts p53 conformation and inhibits its DNA binding activity (378,379).

Anti-oxidants/redox inhibitors

As the present evidence suggests a strong pro-inflammatory role of free radicals, administration of high dose anti-oxidants should be an attractive therapy in patients with RA.

Anti-oxidants can also interfere with signaling pathways (e.g. NF-κB activation) independently of ROS (380). However, although high dosages of vitamin E and vitamin C have demonstrated beneficial effects in animal models of RA (381,382), most placebo-controlled studies with nutrient supplements containing antioxidants in RA patients have been disap-
pointing. The antiarthritic and anti-inflammatory potential of N-acetyl-L-cysteine (NAC) is more established (reviewed in (383)).

Glutathione-synthetase rapidly converges NAC into GSH, the main intracellular redox regulator, hence strongly increasing its anti-oxidative potential and blocking ROS dependent signaling pathways. NAC was tested in CIA and showed significant reduction of arthritis (384,385). Other compounds that increase intracellular GSH are expected to have similar effects. In particular, KE-298/KE-758 drastically increased the level of intracellular GSH, similar to high concentrations of NAC, which may explain its efficacy in rat AA and cases of RA (386-388). On the other hand, changing the cellular redox state in the opposite direction, could also have beneficial therapeutic effects, as stressed by the dual regulation of most signaling pathways. An example is the low molecular weight thioredoxin blocker MOL-294 that inhibits both NF-κB and AP-1 transcription in a mouse model of asthma (389). This compound could potentially be very effective in reducing the severity of inflammation and joint destruction in arthritis as it modulates two important transcription factors.

Inhibition of ROS producing enzymes

Although scavenging free radicals with anti-oxidants could have beneficial effects, more attractive, specific therapeutic options lie in the inhibition of ROS producing enzymes.

NADPH oxidase

Two inhibitors of NADPH oxidase have been tested in animal models of arthritis: diphenylene iodonium chloride (DPI) and staurosporine. They were tested in male DBA/1xB10A(4R) hybrid mice suffering from potassium peroxochromate-induced arthritis. In these settings daily doses of 2.8 μmol/kg of DPI or 30 nmol/kg of staurosporine sufficed to inhibit the arthritis by 50%. A complete inhibition was obtained with 10 μmol/kg of DPI, and 100 nmol/kg of staurosporine suppressed the arthritis by 85% (390,391). However, the involvement of NADPH oxidase in RA is probably far more complex and oxygen radicals might also be important in controlling disease severity, as NADPH oxidase-deficient mice were shown to suffer from more severe arthritis (392).

NO Synthase (NOS)

One of the key free radicals in chronic inflammation is NO. N-(G)-monomethyl-L-arginine (L-NMMA), a competitive nonspecific inhibitor of both constitutive and inducible isoforms NOS, prevented intraarticular accumulation of leukocytes, joint swelling, and bone erosion in animal models of RA (393,394). However, in some studies NOS inhibition only reduced AA if injected before or close to the time of adjuvant application, but not if administered after the establishment of inflammation (393,395). This suggests that NO may be involved in the initial stages of RA but not in the maintenance of chronic inflammation and subsequent joint destruction.
Three structurally distinct isoforms of NOS have been identified: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). Accordingly, selective inhibition of nNOS, iNOS or eNOS may provide a novel therapeutic approach to rheumatoid arthritis. For these reasons, the identification of N-methyl-L-arginine (L-NMA) as the first inhibitor of NO biosynthesis, led to high expectations of their therapeutic potential in RA. In arthritic rats however, selective inhibition of inducible nitric oxide synthase exacerbated erosive joint disease (396).

Xanthine oxidase (XO)
The activity of XO is increased up to fifty-fold in the serum of RA patients compared with healthy controls or patients with other disorders (397). Although the inhibition of xanthine oxidase by allopurinol has been shown to reduce oxidative burst in leucocytes, no beneficial effects have been demonstrated in patients with RA (398).

Antirheumatic drugs and ROS
The therapeutic effects of gold have been attributed to enzymes involved in both the generation and/or scavenging of ROS (399). While the mechanism of the anti-rheumatoid activity of gold compounds remains obscure, it has also been suggested that locally-produced free radicals could oxidize gold to Au(III) or aurocyanide, both of which are highly toxic and could kill or damage activated white cells (400). D-penicillamine could exert its therapeutic effects by forming a complex with copper, then acting as a superoxide dismutase mimetic (401). D-Penicillamine also scavenges hydrogen peroxide and hypochlorous acid and suppresses the stimulated release of ROS from human neutrophils (402). More recently, it has been suggested that the anti-inflammatory actions of methotrexate could also be dependent on ROS metabolism. (403,404).

In conclusion, much more basic research is required to finely elucidate the molecular pathways regulating ROS production in RA. This is illustrated by a recent report suggesting that endogenous ROS do not induce NF-κB activation, but actually decrease NF-κB activity and anti-oxidants also exert effects independently from ROS, for example by lowering the affinity of receptor to TNFα (380). Nevertheless, interfering with intracellular ROS metabolism could certainly prove to be an attractive tool in manipulating cellular signaling in chronic inflammatory diseases such as RA.

CONCLUSIONS

In summary, we have outlined the major signal transduction pathways identified to date in the pathogenesis of RA, and the possible ways of interfering with these pathways via small molecule inhibitors or gene therapy. The publications and patents summarized in this review
only partially represent all of the compounds that are currently being developed and tested in arthritis. However, we have chosen to include only the relatively specific inhibitors with disclosed pharmacological data. The vast amount of scientific literature on signal transduction in RA, especially on NF-κB, and inhibitors of signaling molecules, suggests that signal transduction pathways and transcription factors represent very promising targets for treating this disease as well as other chronic inflammatory disorders that are linked to altered cellular signaling.

Because of the complex interactions that exist between signal transduction pathways in general, there is a need for increasing knowledge about the precise function of individual signaling molecules both in normal physiology and pathophysiology. Consequently, newer generation signaling component inhibitors will need to display increasing specificity, to exert maximal efficacy with minimal side effects. Given the recent progress in the development of specific inhibitors and the fact that some of these compounds have already been tested in Phase I and/or Phase II studies, such targeted therapies are likely to prove useful in the therapy of RA and other chronic inflammatory diseases in the near future.

In addition to the strategies reviewed in this paper, the advent of new techniques to interfere with signal transduction pathways will certainly provide the tools for even more specific blockade of signaling molecules, resulting in precisely defined biological effects. Examples of these techniques are small interfering RNA (siRNA), HDAC inhibitors, and cell type-specific peptides that affect distinct signaling molecules. The ultimate benefit of targeting signal transduction will depend on the delicate balance between suppressing inflammation and interfering with normal cellular functions. By using local gene therapy or intralesional administration of targeted small molecules, this goal appears attainable.
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Chapter 3
Concise communication

Reduction of pro-inflammatory cytokine expression in the synovium by targeting inhibitor of nuclear factor (NF)-κB kinase β in vivo

Sander W. Tas¹, Najat Hajji¹, Dirk J. Stenvers¹, Gary S. Firestein², Margriet J. Vervoordeldonk³, and Paul P. Tak¹

¹Div. of Clinical Immunology & Rheumatology, AMC/University of Amsterdam, The Netherlands.
²Div. Of Rheumatology, Allergy, and Immunology, University of California, San Diego School of Medicine, La Jolla, CA and ³Arthrogen BV, Amsterdam, The Netherlands
Nuclear factor (NF)-κB is one of the most important pro-inflammatory transcription factor families involved in the pathogenesis of rheumatoid arthritis (RA) and contributes significantly to synovial inflammation (1-3). NF-κB activation can be induced by many different stimuli and is well controlled by various endogenous mechanisms, including the inhibitory κB (IκB) proteins that are under tight control of the IκB kinase (IKK) complex. Under inflammatory conditions NF-κB is mainly activated via this canonical signal transduction pathway (reviewed in (4)). We recently established that IKKβ is a key regulator of synovial inflammation and demonstrated that intra-articular (i.a.) administration of a dominant negative form of IKKβ using adenoviral gene therapy (Ad.IKKβdn) significantly reduced clinical signs of arthritis activity (5). The consequences of local IKKβ inhibition on synovial cytokine expression at the site of inflammation have as yet not been studied in in vivo models of RA.

The present study was conducted to provide more insight into the mechanism of action of Ad.IKKβdn gene therapy. Therefore, we investigated the consequences of local inhibition of IKKβ on synovial cellularity, cytokine expression and the expression of matrix degrading enzymes. Serial sections from paraffin-embedded ankle joints of rats with adjuvant arthritis (AA) treated with intra-articular Ad.IKKβdn gene therapy (n=10) were analyzed and compared to Ad.GFP injected control animals (n=10) (5). After blocking endogenous peroxidase activity (hydrogen peroxide) and antigen retrieval (citrate buffer), sections were incubated overnight at 4°C with antibodies specific for rat IL-1β, TNFα, IL-6 and IL-10 in PBS/BSA 1% (10 μg/ml; all from R&D Systems). In addition, immunohistochemical stainings were carried out to detect matrix-metalloproteinase (MMP)-3 (10 μg/ml; Chemicon, Temecula, CA) and tissue inhibitor of MMP (TIMP)-1 (5 μg/ml; R&D Systems). After incubation with secondary HRP-conjugated swine-anti-goat or goat-anti-mouse antibodies (Dako, Glostrup, Denmark) in PBS/BSA 1%, signal amplification was performed using biotinylated tyramine (Perkin Elmer Life Sciences) followed by streptavidine-HRP (Dako) in PBS/BSA 1% as described previously (6). Finally, peroxidase activity was detected using AEC (0.02% 3-amino-9-ethylcarbazole; Vector Laboratories, Peterborough, UK) as dye and hydrogen peroxide as substrate. Sections were counterstained with Mayer’s haemalum solution (Merck, Darmstadt, Germany) and mounted with Kaiser’s glycerol gelatin (Merck) mounting medium. Computerized image analysis was used to evaluate stained sections in a random order (7). Results were expressed as integrated optical density (IOD)/mm², which is proportional to the cellular concentration of protein multiplied by the area of positive staining. Data were analyzed for statistical significance (SPSS 11.5.1 Statistics UK) using the Mann-Whitney U test and a p value < 0.05 was taken as the level of significance.

Intra-articular Ad.IKKβdn gene therapy resulted in a 50% reduction of synovial cellularity (104 ± 23 vs. 212 ± 35 cells/mm², IKKβdn vs.GFP, respectively; p < 0.05). In addition, we observed significantly reduced synovial IL-1β and TNFα expression in Ad.IKKβdn treated rats (Figure
Figure 1. Reduced synovial pro-inflammatory cytokine and MMP3 expression in Ad.IKKβdn treated rats. See Color figures.
Expression of IL-1β, TNFα, IL-6, IL-10, MMP3 and TIMP-1 in arthritic ankle joints of rats treated with Ad.IKKβdn. Representative pictures of synovial cytokine staining are shown (overview + detailed image of TNFα expression), followed by results from digital image analysis of the immunohistochemical stainings (n=10/group). Data are expressed as IOD ± SEM (* P < 0.05).
Reduction of pro-inflammatory synovial cytokine expression by Ad.IKKβdn gene therapy

Expression of IL-6 was also reduced in the Ad.IKKβdn treated group (Figure 1), although this did not reach statistical significance. Interestingly, expression of the anti-inflammatory cytokine IL-10 was not altered by Ad.IKKβdn treatment (Figure 1). These data are in agreement with the recognized clinical efficacy of Ad.IKKβ gene therapy in animal models of RA (5). Next, we investigated the effects of Ad.IKKβdn gene therapy on the expression of MMP-3 and TIMP-1 in synovial tissue. Local IKKβ inhibition resulted in significantly decreased MMP-3 expression (Figure 1; P < 0.05), whereas expression of TIMP-1 was unaffected (Figure 1).

All known effective anti-rheumatic therapies result in decreased cellularity of rheumatoid synovial tissue, and for that reason synovial cellularity is used as a sensitive biomarker for the evaluation of novel therapies (8,9). Of importance, the sensitivity to change of key synovial biomarkers is high in RA patients receiving active treatment (9). The results presented here, using computer assisted image analysis, show that intra-articular Ad.IKKβdn gene therapy results in a dramatic reduction of synovial cellularity, supporting the notion that this may be an effective approach to treat arthritis.

TNFα and IL-1β are cytokines that play an important role in the pathogenesis of RA and targeting these cytokines has been proven effective in reducing arthritis severity (10). Conversely, the expression of TNFα and IL-1β is greatly reduced in rheumatoid synovial tissue after treatment with effective anti-rheumatic therapies like prednisolone (8). The significantly reduced synovial IL-1β and TNFα expression in Ad.IKKβdn treated rats is consistent with the clinical efficacy of Ad.IKKβ gene therapy in AA (5). Furthermore, in vitro experiments have demonstrated that IKKβ is essential for IL-1β production by synovial membrane cells and TNFα production by macrophages following CD40L stimulation (11). Although not significantly, IL-6 expression was reduced after Ad.IKKβdn gene therapy, which is in line with the fact that the IL-6 promoter has DNA binding sites for several transcription factors including NF-κB (12). Thus, local IKKβ inhibition in vivo results in a reduction of three known pro-inflammatory cytokines involved in RA. Importantly, expression of the anti-inflammatory cytokine IL-10 was not altered by Ad.IKKβdn treatment, which is consistent with the lack of NF-κB/Rel responsive promoters in the IL-10 gene (13). This has important implications, since IKKβ inhibition selectively blocked the expression of pro-inflammatory cytokines, whereas the levels of the anti-inflammatory cytokine IL-10 were not affected. Collectively, these data indicate that local canonical NF-κB inhibition, in addition to reducing synovial cellularity, also results in an anti-inflammatory shift in synovial cytokine expression, which may largely explain the beneficial effects of Ad.IKKβdn gene therapy on arthritis severity.

Matrix degradation and erosion of the connective tissue start at sites of attachment of synoviocytes to cartilage (14). At the invasive front, the synovial fibroblasts are found to express
high levels of MMPs, such as MMP-1 (collagenase) and MMP-3 (stromelysin) (15). The activity of these enzymes is tightly regulated on a transcriptional level by AP-1 and NF-κB responsive promoters (3,16), as well as by interactions with specific inhibitors of the enzymatic activity at the posttranslational level (17). TIMP-1 strongly binds to both MMP-1 and MMP-3 and is, like MMP-1 and MMP-3, produced by synovial fibroblasts at sites of synovial attachment to cartilage (17). Although intra-articular injection of Ad.IKKβdn resulted in significantly decreased MMP-3 expression, this did not result in reduced bone erosion in our previous study (5). This may be due to the fact that MMP expression is also to a large extent dependent on activation of the transcription factor activator protein (AP)-1 (3), which is thought to peak earlier than NF-κB in arthritis. Ad.IKKβdn gene therapy may therefore have been applied too late to prevent early MMP-induced bone destruction. Alternatively, intra-articular treatment with Ad.IKKβdn could result in biologically insufficient reduction of total MMP levels to prevent bone destruction in this severe animal model of RA. TIMP-1 expression was not significantly increased in synovial tissue of Ad.IKKβdn treated rats, which is in line with the fact that no NF-κB responsive elements in the promoter region of the TIMP-1 gene have been described (18-20).

The present data validate earlier in vitro experiments using an adenoviral technique of blocking NF-κB in RA synovial cells through overexpression of IκBα or IKKβdn (11,21). Our findings demonstrate that selective inhibition of the canonical NF-κB pathway in vivo using Ad.IKKβdn gene therapy results in decreased expression of pro-inflammatory cytokines and destructive MMP-3, while expression of anti-inflammatory IL-10 and TIMP-1 was not affected. Detailed immunohistochemical analysis of the synovial tissue results in a better understanding of the underlying mechanisms of clinical efficacy. The data presented here support the notion that intra-articular IKKβdn gene therapy may be effective as a novel approach to treat RA (5).
Reduction of pro-inflammatory synovial cytokine expression by Ad.IKKβdn gene therapy

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Chapter 4

Enhanced gene transfer to arthritic joints using adeno-associated virus type 5: implications for intra-articular gene therapy

Adriaansen J1, Tas SW1, Klarenbeek PL1, Bakker AC2, Apparailly F3, Firestein GS4, Jorgensen C5, Vervoordeldonk MJBM1, Tak PP1

1Div. of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. 2Amsterdam Molecular Therapeutics, Amsterdam, The Netherlands. 3Unité de recherches d’immunopathologie des maladies tumorales et auto-immunes INSERM U475, Montpellier, France. 4Division of Rheumatology, Allergy, and Immunology, University of California San Diego, La Jolla, California, USA. 5Service d’Immunorhumatologie, CHU Lapeyronie, Montpellier, France

ABSTRACT

Background: Gene therapy of the joint has great potential as a novel therapeutic approach for the treatment of rheumatoid arthritis. The vector of choice is of crucial importance for clinical success.

Objective: To investigate the tropism and transduction efficiency in arthritic joints in vivo, and in synovial cells in vitro, using five different serotypes of recombinant adeno-associated virus (rAAV) encoding β-galactosidase or green fluorescent protein genes.

Methods: Recombinant AAV was injected into the ankle joints of rats with adjuvant arthritis after the onset of disease. Synovial tissue was examined at different time points for β-galactosidase protein and gene expression by in situ staining and PCR analysis, respectively. In addition, the capacity of rAAV to transduce primary human fibroblast-like synoviocytes from rheumatoid arthritis (RA) patients was investigated in vitro.

Results: We found that intra-articular injection of rAAV serotype 5 resulted in the highest synovial transduction, followed by much lower expression using rAAV2. The expression of the transgene was already detectable 7 days after injection and lasted for at least 4 weeks. Only background staining was observed for serotypes 1, 3 and 4. Importantly, there was a minimal humoral immune response to rAAV5 compared to rAAV2. In addition, we found that both rAAV2 and rAAV5 can efficiently transduce human fibroblast-like synoviocytes obtained from RA patients.

Conclusion: These data indicate that intra-articular rAAV-mediated gene therapy in RA might be improved by using rAAV5 rather than other serotypes.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent joint swelling and progressive destruction of cartilage and bone. Biologicals targeting cytokines, like TNF-α or IL-1, and their receptors have proven useful as specific therapies for RA (1-4). However, the need for continuous treatment with multiple injections of a recombinant protein, the relatively high systemic doses necessary to achieve constant therapeutic levels in the joints, and the reported side effects hamper this therapeutic approach.

An alternative approach could be to use intra-articular gene therapy to deliver therapeutic genes directly to the synovium in order to have the protein synthesized at the site of inflammation. Synoviocytes can be readily accessed by intra-articular injection, making them good targets for gene delivery. They are resident cells in the human synovium and have a low mitotic rate (5). Therefore, they are likely to express transduced genes for a considerable length of time.

Viral-mediated gene transfer is currently the most efficient system for delivering therapeutic proteins in vivo. Adeno-associated virus (AAV) especially holds great promise as a novel vector and appears to be safe (6,7). AAV is a single-stranded DNA virus that does not induce a significant immune response and is not associated with disease in humans. The capacity of AAV to mediate expression of therapeutic genes has been well-established in several animal models of arthritis (8-12). Recombinant AAV2 (rAAV2) has been validated for gene therapy in human clinical trials in cystic fibrosis, α-1-antitrypsin deficiency, and hemophilia (13-15). rAAV vectors are particularly useful in targeting slowly dividing cells and in the treatment of chronic disease because of their potential for site-specific integration into the host genome or formation of stable episomal DNA, both of which result in long-term gene expression (16,17).

All studies reported so far have been performed using rAAV serotype 2. However, rAAV2 vectors are rather inefficient in transducing some cell types (18-20). In addition, the prevalence of antibodies against this serotype is high in the human population (6). Many individuals carry antibodies that are able to neutralize infection of cells with rAAV2 in vitro, and it is widely assumed that this is also relevant in vivo (21,22). At this moment at least eight naturally occurring serotypes of AAV have been isolated, cloned and sequenced (AAV1 to AAV8). Although these serotypes are very similar in genetic structure and biological properties, the capsid genes show as little as 50-60% homology in DNA sequence, resulting in viral shells that have a different amino acid composition (6,23). The use of non-type 2 AAV capsids to deliver transgenes to target cells might offer two important advantages over conventional AAV2 vectors: 1) an overall broader host range, and 2) an escape from anti-AAV2 immune responses, allowing vector (re-) administration despite existing immunity against AAV2.

In an effort to compare the role of serotype-specific virion shells on synovial transduction, we cloned the serotype capsid encoding domains into a common vector backbone containing AAV2 replication genes. This strategy allows the packaging of AAV2 inverted terminal
repeat (ITR) vectors into each serotype’s specific virions, resulting in hybrid serotype vectors differing only in their capsids. In the study presented here we compared the transduction efficiency of five different recombinant AAV serotypes (rAAV1 to rAAV5) encoding the E. coli derived reporter gene, β-galactosidase (β-Gal) to transduce arthritic synovium in vivo. In addition, we examined the transduction efficacy of rAAV containing the enhanced green fluorescent protein gene (GFP) in primary human RA fibroblast-like synoviocytes (FLS).

MATERIAL AND METHODS

Production of recombinant AAV

All rAAV constructs were derived from AAV2 and driven by the cytomegalovirus immediate early (CMV) promoter. Recombinant AAV was produced by co-transfection of 293 HEK cells with 150 μg packaging plasmid (pDG for AAV2 (24) and pDP1, pDP3, pDP4 and pDP5 for AAV1, AAV3, AAV4 and AAV5 (25), respectively) and 50 μg vector plasmid containing the LacZ or the GFP gene with the CMV promoter and the bovine growth hormone polyA region between the AAV2 ITR’s, by the calcium phosphate method. Plasmids were purified with Qiafilter plasmid Giga Kits (Qiagen, Alameda, USA). 72 h post-transfection, the cells were lysed using benzonase (Merck, Whitehouse Station, USA) to a final concentration of 75 U/ml. Crude cell lysates were further purified with iodixanol gradients as described earlier (26). The gradient layer containing the virus was extracted, diluted 10 times with PBS and concentrated to approximately 2 ml with centricon devices (YM-100, Millipore Corporation, Billerica, USA). Stock viral titers ranging between 10^{11}-10^{12} genomic copies (GC)/ml in saline were reached.

Detection of viral genomic copies by Quantitative-PCR

To determine the viral titre in terms of genomic copies (full viral particles), rAAV samples were first diluted 10-fold in PBS. Samples were incubated with 10 μl of MagneSil BLUE suspension (Promega) and the viral DNA was isolated using the MagnaBot 96 Magnetic Separation Device (Promega) according to the supplier’s instructions. Dilutions of the purified viral DNA or the gDNA isolated from the joints and organs were added to PCR mix containing 0.5 μM of the CMV forward primer (S’AATGGCGCGGTAGCGCTGTTA3’) (Invitrogen), 0.5 μM of the CMV reverse primer (S’AGGCGATCTGACGGTTCACTAA3’) (Invitrogen), and SYBR green PCR master mix buffer (Applied Biosystems, Foster City, USA). PCR reactions were performed using the Abi prism SDS7000 sequence detection system (Applied Biosystems, Foster City, USA).

Animals

Pathogen-free male Lewis rats (150-200 g) were obtained from Harlan Sprague Dawley Inc. (Horst, The Netherlands) and were maintained in our central animal facility. The Ethical Animal Care and Use Committee of the University of Amsterdam approved all experiments.
Local gene transfer

All rats were immunized at the base of the tail with 1 mg of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, USA) in 0.1 ml mineral oil on day 0 (27). Paw swelling was usually observed by day 10-12 and measured daily by water displacement plethysmometry. At day 12, the skin was disinfected with ethanol and 6.1 x10^10 GC rAAV1-5 containing the gene encoding LacZ (further referred to as rAAV1-5) were injected anterolaterally into the right ankle joint in a total volume of 50 μl saline using a 31-gauge needle on a glass syringe (28). Adenovirus containing the LacZ gene (adjusted to 6.1 x10^10 GC/animal) served as a positive control and diluent was used as negative control. rAAV injected and control rats were sacrificed two weeks after intra-articular injection by CO₂ inhalation, whereas adenovirus-injected rats were sacrificed two days after injection (n=6/group). Serum samples were taken from the vena cava. Hind paws, spleen, heart, lung, liver, and kidney were collected.

To investigate the transgene expression at different time points, a second experiment was performed using a different batch of rAAV. Animals received an intra-articular injection of 1.14 x10^10 GCs rAAV2 or rAAV5 and were sacrificed as described one, two, three and four weeks after injection (n=3/group). Serum samples were obtained from all groups by tail bleeding before rAAV injection and by vena cava puncture during sacrificing.

Detection of β-galactosidase activity

Joints were fixed in 4% formalin, decalcified using EDTA and snap frozen in liquid nitrogen. Ten μm sections were cut in on cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Germany). Subsequently, detection of β-Gal activity was performed by X-Gal staining as described previously (28). After washing with PBS, sections were counterstained with nuclear red. The sections were evaluated by digital image analysis.

Digital image analysis

Five randomly selected fields within each section were chosen for digitizing the positive signal. These images were acquired on an Olympus microscope (Olympus, Tokyo, Japan), captured using a Charged Coupled Device video camera (Sony, Tokyo, Japan) and digitized with a PV100 multimedia 16-bit color video digitizer card. In the resultant color images the area of positive staining and the mean optical density (MOD) were measured by a macro program as described earlier (29,30). The MOD is proportional to the cellular concentration of protein. The integrated optical density (IOD) is equal to the MOD multiplied by the area of positive staining.

Real Time-PCR detection of LacZ

Ankle joints (trimmed of skin) and organs were snap frozen in liquid nitrogen, pulverized using a mortar, and homogenized in TRIzol Reagent (100 mg/ml) (GibcoBRL Life Technologies, Gaithersburg, USA) using a tissue homogenizer. Total RNA was isolated from the aqueous
phase and genomic DNA (gDNA) was extracted from the phenol-chloroform phase according to the manufacturer's instructions. gDNA was stored for Quantitative-PCR (Q-PCR) analysis. RNA was dissolved in DEPC-water and cDNA was synthesized from 1 μg RNA using SuperScript II RT (Invitrogen) according to the manufacturer's instructions.

For RT-PCR, 10 μl of cDNA solution was amplified using 25 μl of AccuPrime SuperMix I (Invitrogen), 215 mmol of the LacZ forward primer (5'-GCATCGAGCTGGTAATAAGCGTTGGCAAT-3') and 215 mmol of the LacZ reverse primer (5'-GACACCAGACCAACTGGTAATGGTAGCGAC-3') in a total volume of 50μl. Rat GAPDH was used as an internal control (forward: 5'-CGGTGTCAACGGATTTGGC-3', reverse: 5'-CCATGCCAGTGAGCTTCCC-3'). Amplification was then performed in a thermocycler (MJ Research, Inc, Waltham, USA) as follows: 3 min at 95°C followed by 35 cycles of 94°C for 1 min, 58°C for 90 sec and 72°C for 1 min, respectively, followed by a final extension phase at 72°C for 10 minutes. The PCR products were analyzed by standard agarose gel electrophoresis on a 0.9% agarose gel containing ethidium bromide for UV-assisted visualization of the 622 bp product. For semi-quantitative analysis, the gel was digitized and the signal was corrected for background and area. Thereafter, the LacZ/GAPDH ratio was determined using Quantity One software (Biorad, Hercules, USA).

Formation of anti-rAAV neutralizing antibodies in serum
Neutralizing antibody titers were analyzed by assessing the ability of serum antibodies to inhibit the transduction of rAAV into COS cells. Various dilutions of serum (1:200 to 1:51200) were pre-incubated with rAAV at 37°C for 1 hour and then added to 80% confluent COS cells. Thereafter, cell cultures were incubated with rAAV in the presence of serum for 20 hours and LacZ expression was measured by β-Gal staining as described earlier. The antibody titer was represented by the highest dilution that did not exhibit inhibition of β-Gal expression compared to cells incubated with rAAV alone.

In vitro transduction of human fibroblast-like synoviocytes
Small-bore arthroscopy (2.7mm arthroscope, Storz, Tuttlingen, Germany) was performed under local anesthesia in patients with established RA (n=3) (31,32).

The biopsies were enzymatically dispersed and cultured as described previously (33) and the obtained FLS were plated on 6-wells dishes (Flacon, Bedford, USA) at 1 x10^5/well. After incubation for 15 hours, 1 x10^8 GCs of rAAV1-5 containing the gene for GFP was added to each well in medium containing 10% FCS. The cells were cultured for 72 hours and marker gene expression was evaluated by fluorescent microscopy and flow cytometric analysis. Three independent primary FLS cell lines from three patients were used for these experiments.

Flow cytometry
Transduced FLS were trypsinized, resuspended in DMEM containing 10% FCS and washed in PBS containing 0.01% (w/v) NaN_3 and 0.5% (w/v) bovine serum albumin. Analysis of GFP
expression was performed using a FACS Calibur flowcytometer and CellQuest software (BD Biosciences, Franklin Lakes, USA).

Statistics
Where applicable, differences in means between groups were determined by Kruskal-Wallis test, followed by a Mann-Whitney U rank-um test. p<0.05 was considered statistically significant. All analysis were done using SPSS version 11.5 (SPSS, Chicago US)

RESULTS

Comparative efficiency of five rAAV serotypes
To compare the transduction efficiency of rAAV serotypes in the joints, rAAV1-5 were injected into the right ankle joints of rats (n=6/group) on day 12 after adjuvant immunization. Joints were harvested two weeks after injection and stained in situ to measure β-Gal expression. Staining was quantified by digital image analysis. The strongest expression of β-Gal in synovial tissue was detected in arthritic joints injected with rAAV5, followed by a much lower expression using rAAV2 (Figure 1). Strikingly, rAAV transduction resulted in a greater penetration into the synovial tissue compared to adenovirus, where β-Gal expression was limited to the intimal lining layer. In the control joints no staining could be observed (Figure 1A). No expression above background staining was observed for serotypes 1, 3 and 4. These findings were confirmed by Q-PCR. In genomic DNA isolated from crushed joints, the highest number of viral genomic copies was detected using rAAV5 (1.9x10^5 GC/μg gDNA) (Figure 2). Semi-quantitative RT-PCR showed the presence of LacZ mRNA in all injected joints, but not in spleen, liver, heart, lung, kidney or uninjected joints (data not shown).

Duration of transgene expression
To monitor the transgene expression over time, rAAV2 or rAAV5 were injected into arthritic joints of rats and sacrificed one, two, three or four weeks after injection of the vector. For both serotypes transgene expression was detectable one week after injection with marked

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<th>Table 1. Detection of viral genomic copies in injected joints after intra-articular injection of AAV2 or AAV5.</th>
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Genomic DNA was isolated from crushed ankle joints 1, 2, 3 or 4 weeks after injection. Q-PCR was performed with specific primers for the CMV promoter of the transgene. Values are expressed as GC/μg gDNA ± SD.
Figure 1. β-gal expression in rat synovial tissue 2 weeks after i.a. injection of different rAAV serotypes. See Color figures.

Joints were snap frozen and sections were cut and stained in situ for β-Gal activity and counterstained with nuclear red. Representative cryosections of right ankle joints of rats with AA, injected with $6.1 \times 10^{10}$ GC rAAV, Ad.LacZ as a positive control or saline as a negative control are shown (magnification 100x, 400x). The lining layer is indicated by arrows (A). Tissue sections were quantified for β-Gal expression by computer assisted digital image analysis (B). Values are expressed as cumulated IOD per mm² (IOD: Integrated Optical Density) ± s.d. * Statistically different using the Mann-Whitney U test with P-values <0.05 as compared to the control group.
expression for up to four weeks, but displayed different expression kinetics. rAAV5 resulted in earlier β-Gal expression than rAAV2, with higher levels at all time points (Figure 3). This was confirmed by Q-PCR analysis. The number of genomic copies detected in the joints after rAAV5 injection was higher at all time points compared with rAAV2 (Table 1).

To test if this increased amount of viral genomic copies also results in an increased transcription of the transgene, RT-PCR analysis was carried out using specific primers for LacZ. Figure 4 demonstrates that in rAAV2 as well as in rAAV5 injected animals, high levels of LacZ mRNA were found after one week. This rapidly declined in the rAAV2 injected animals, whereas in the rAAV5 injected animals the level of LacZ mRNA remained about 50% of the levels found after one week.
A limited (n=3/group) biodistribution study was performed and no viral DNA or LacZ mRNA was found in the uninjected left joints at any time point. A relatively low number of GCs was found only in kidney and liver after one week, but not at later time points suggesting minimal, temporary dissemination of the vector (data not shown). In spleen, liver, heart, lung, kidney and lymph nodes, no LacZ mRNA was detected at any time point (data not shown).

Formation of rAAV antibodies
To detect a possible humoral immune response against the rAAV capsid proteins after intra-articular injection, we performed a neutralizing antibody assay. The presence of neutralizing antibodies before and after rAAV injection was determined in the serum of rats injected with rAAV2 and 5. Before injection, no antibodies were found in any of the samples. One week after injection, neutralizing antibodies were detected, peaking at 2 weeks and slowly decreasing after 3 weeks. Although this tendency was seen for both serotypes, rAAV2 injection obviously
induced higher neutralizing antibody titers in serum than rAAV5, which induced titers only slightly above background (Figure 5). Importantly, no cross-reactivity was found for the two serotypes (data not shown).

Transduction of human fibroblast-like synoviocytes

Having shown that rAAV2 and 5 are able to transduce rat synovium, we investigated the potential of both serotypes to transduce primary human FLS obtained from patients with RA. For this purpose we used rAAV vectors expressing GFP. FLS were incubated with the different rAAV serotypes and transgene expression was visualized after 72 hours by fluorescent microscopy (Figure 6). Both serotypes were capable of transducing human FLS with high efficiency, although no clear difference was seen between rAAV2 and rAAV5. To quantify the percentage of GFP expressing cells, FLS were transduced using five rAAV serotypes as described and GFP expression was evaluated by FACS analysis (Figure 7). Both rAAV2 and
rAAV5 resulted in significant numbers of GFP expressing cells (45% and 40%, respectively), compared to control (Figure 7A). Compared to rAAV1, 3 and 4, rAAV2 and rAAV5 transduced significantly more FLS (Figure 7B), p<0.05.
DISCUSSION

Recombinant AAV vectors have gained much attention due to their ability to mediate efficient transduction of both dividing and non-dividing cells and their capability to induce long-term gene expression in the absence of toxicity. Over the last decade, rAAV vectors derived predominantly from serotype 2 were generated and investigated in vivo. The recent discovery of AAVs other than serotype 2 has enabled the generation of pseudotyped rAAV vectors. Until now, no gene transfer studies to the inflamed joint have been carried out with non-serotype-2 vectors. Therefore, we compared the transduction efficiencies of titer-matched rAAVs derived from serotypes 1 to 5, in synovial tissue of rats with AA. The efficiency of gene expression was evaluated by enzymatic staining and by determining the amount of genomic copies (GCs) present in the joints. In this comparative study we found that direct injection of rAAV5 into the ankle joints of rats with AA resulted in the highest synovial transduction as demonstrated by the expression level of β-Gal, followed by a much lower expression using rAAV2. Analysis of the gDNA isolated from the joints, confirmed these results. In a second experiment we investigated the transgene expression of rAAV2 and rAAV5 at different time points. Both serotypes demonstrated marked expression over time, which was already present one week after injection. Of importance, β-Gal protein expression using rAAV5 was higher at all time points, confirmed by higher levels of mRNA encoding β-Gal. The fact that the genomic DNA profile did not parallel β-Gal expression in time could be explained in part by the relative stability of the β-Gal protein, resulting in prolonged presence of the protein. Although β-Gal is a foreign protein and possibly immunogenic, we did not observe differences in paw swelling or cellularity between rAAV injected and control animals (data not shown).

Recombinant AAV2 has previously been shown to mediate long-term gene transfer in a number of tissues, including synovial tissue (8-10,34-36). The feasibility of direct in vivo gene transfer to rat and murine arthritic joints has also been demonstrated. In most of the studies, rAAV2 genome persisted within joints for at least 100-200 days (8,11,36). One study reported long-term expression after rAAV2 gene transfer to the murine synovium for up to 7 months (9). The expression pattern of rAAV2 following intra-articular injection in mice has been variable, varying from synovial lining cells (36) to chondrocytes (11), and myocytes (8,9). In our study, morphological analysis suggested that the β-Gal expressing cells were mainly synovial fibroblasts. Since these cells are thought to stay in the synovial tissue for long periods of time, this would allow long-term transgene expression in the joint, with minimal spreading of transduced cells to peripheral tissues. In addition to the intimal lining layer, cells in the synovial sublining were transduced as well, maximizing the potential of therapeutic protein production throughout the synovial tissue.

Serotype-specific affinities for cell surface attachment and internalization receptors may at least in part account for the difference in the level of transgene expression between the different serotypes. Heparan sulfate proteoglycans (37), integrins (38) and fibroblast growth
factor receptor 1 (39) are important receptors for rAAV2. Recently, N-linked sialic acid (40) and platelet derived growth factor receptor (PDGFR) (41) were identified as co-receptors for AAV5, and their expression in vivo correlates well with the transduction profile of rAAV5 (41). In addition, trafficking of rAAV5 to the nucleus or its uncoating could be more efficient than for the other serotypes, explaining the higher number of GCs and gene expression observed with rAAV5.

Having shown the feasibility of direct in vivo gene transfer to rat synovium, we investigated the transduction efficiency of rAAV to human FLS derived from patients with RA, which is crucial for using this vector for local human gene therapy. FLS are the most resident cells in the human synovium and have a low mitotic rate (5,42). Therefore, FLS are likely to express transduced genes for a considerable length of time, even if the transgene is located episomally. In contrast to rAAV1, 3, and 4, synovial fibroblasts isolated from the joints of RA patients could be transduced with high efficiency in vitro using both rAAV2 and rAAV5. No significant differences were observed between these two serotypes, suggesting that factors other than differences in affinity for cell surface receptors contribute to the different tropism seen in vivo. The transduction of human macrophages was most efficient using rAAV5, but resulted in only a low percentage of GFP positive cells (data not shown).

Some studies investigating the effect of repeated administration of rAAV indicate that an immune response generated after the first administration may prevent retreatment (43). We found that local rAAV2 injection in the joint clearly induces more neutralizing antibodies in rat serum than rAAV5, which induced neutralizing Abs levels only slightly above background. No antibodies were detected before injection of the vectors. This indicates that a marked humoral immune response is mounted against rAAV2, while the capsid of rAAV5 is less immunogenic. Importantly, no cross-reactivity was observed between antibodies raised against rAAV2 and rAAV5, suggesting that re-administration could be applied using different serotypes. It should be noted that an estimated 80% of the human population carry antibodies to the capsid proteins of wild-type (wt) AAV2, and 30 to 70% demonstrate the presence of neutralizing anti-capsid antibodies (21,44). In contrast, neutralizing antibodies against AAV5 are rare. Moreover, endogeneous sequences of AAV5 suggesting previous infection with this serotype could not be detected in a large diverse group of individuals (45). Together, these observations are important for the choice of a rAAV serotype vector for clinical applications, favoring the use of rAAV5.

In summary, our results show that rAAV5 is more efficient in transducing rat synovial tissue in vivo and both rAAV2 and rAAV5 can efficiently transduce human RA synoviocytes in vitro. In addition, we showed little to no humoral immune response to rAAV5 in rat AA. From the five rAAV serotypes tested rAAV5 appears to be an excellent candidate vector for local gene therapy in patients with RA, allowing long-term expression of the transgene limited to the synovial compartment.
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Chapter 5

Amelioration of arthritis by intra-articular dominant negative IKKβ gene therapy using adeno-associated virus type 5

Sander W. Tas1, Janik Adriaansen1,2, Najat Hajji1, Andrew C. Bakker3, Gary S. Firestein4, Margriet J. Vervoordeldonk1,2, and Paul P. Tak1

1Div. of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, The Netherlands. 2Arthrogen BV, Amsterdam, The Netherlands. 3Amsterdam Molecular Therapeutics, Amsterdam, The Netherlands. 4Div. of Rheumatology, Allergy, and Immunology, University of California, San Diego School of Medicine, La Jolla, CA.
ABSTRACT

Nuclear factor (NF)-κB is highly activated in synovium of rheumatoid arthritis (RA) patients, and can induce transcription of many pro-inflammatory molecules. Phosphorylation of the inhibitor of κB (IκB) proteins is an important step in NF-κB activation and is under inflammatory conditions predominantly regulated by IκB kinase (IKK). Consequently, specific targeting of IKKβ in the joint using gene therapy presents a sophisticated treatment option for arthritis. In the present study we investigated the effect of inhibiting IKKβ in adjuvant arthritis (AA) in rats using rAAV mediated intra-articular gene therapy. For this purpose rAAV5 containing the dominant-negative IKKβ gene (AAV5.IKKβdn) or control AAV5.eGFP were injected into the right ankle joints. Rats treated with AAV5.IKKβdn in early arthritis exhibited significantly reduced paw swelling (p<0.05). Immunohistochemical analysis of synovial tissue revealed reduced levels of IL-6 (p=0.005) and TNFα (p=0.03), whereas IL-10 levels were not affected. No significant effect was found on cartilage and bone destruction, or on matrix metalloproteinase (MMP)-3 and tissue inhibitor of MMPs (TIMP)-1 expression. Injection of AAV5.IKKβdn in the pre-clinical phase only showed a marginal effect on arthritis. Importantly, in this study we also demonstrate for the first time that our vector is capable of transducing human RA whole synovial tissue biopsies ex vivo, resulting in reduced IL-6 production after TNFα stimulation (p=0.03). In conclusion, we are the first to demonstrate that rAAV5 can be used to successfully deliver a therapeutic gene (IKKβdn) to the synovium, resulting in reduced severity of inflammation in AA in vivo and pro-inflammatory cytokine production in human RA synovial tissue ex vivo. This translational research represents a crucial next step towards the development of gene therapy for application in humans.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting predominantly the joints (1). Activation of the pro-inflammatory transcription factor NF-κB has been demonstrated to contribute significantly to inflammation in chronic diseases like RA (reviewed in (2)). Phosphorylation of inhibitor of κB (IκBα) by the IκB kinase (IKK) complex is a crucial step in NF-κB activation. The IKK complex is a key convergence site of many different stimuli that induce NF-κB activation (3). It contains two catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ. NF-κB activation in response to pro-inflammatory signals is mainly dependent on IKKβ (4). The subsequent polyubiquitination targets IκBα for degradation, releasing NF-κB dimers from the NF-κB-IκBα complex, followed by translocation to the nucleus and binding to κB enhancer elements of target genes. This results in the transcription of pro-inflammatory cytokines, chemokines and adhesion molecules (reviewed in (5)). NF-κB is highly activated in synovial tissue of RA patients (6,7), IKKβ being a key regulator of synovial inflammation (8). Various systemic approaches to specifically inhibit the activation of this transcription factor pharmacologically by targeting the IKK complex have proven very successful in the amelioration of arthritis (9-12).

A particularly interesting alternative approach could be to use intra-articular gene therapy to target IKK locally in the inflamed joint in order to have a therapeutic protein synthesized at the site of inflammation, without the potential dangerous side effects of systemic NF-κB inhibition. We have previously demonstrated in a target validation study that local IKKβdn gene transfer using an adenoviral vector effectively suppressed rat adjuvant arthritis (AA) (8). However, adenoviral vectors may evoke serious host immune responses and as a consequence give only transient expression of the transgene (13). Therefore, novel vectors have been developed and tested for their transduction efficiency as well as the stability and duration of transgene expression. Adeno-associated virus (AAV) has emerged as a potential novel vector that lacks many of the immunogenic characteristics of adenoviral vectors and appears to be safe (14). AAV is a single-stranded DNA virus that does not induce a significant immune response and is not associated with disease in humans. Recently, we compared the efficiency of five different recombinant AAV (rAAV) serotypes (rAAV1-rAAV5) to transduce arthritic synovium in different animal models of arthritis. We demonstrated that rAAV5 is an excellent potential vector for local gene therapy in patients with RA, allowing long-term expression of the transgene limited to the synovial compartment, without developing a humoral immune response to the vector (15,16).

As a next step towards development of gene therapy for RA, we constructed an rAAV5 vector containing the IKKβdn transgene (AAV5.IKKβdn) and investigated the therapeutic potential of intra-articular (i.a.) injection of AAV5.IKKβdn in rat AA. In addition, the potential to transduce hu-
man RA synovial tissue was examined \textit{ex vivo}. Our data demonstrate that local NF-κB blockade by IKKβdn using rAAV5 as a vector results in a significant reduction of synovial inflammation.

\section*{MATERIAL AND METHODS}

\subsection*{Animals}
Pathogen-free male Lewis rats (150-200 g) were obtained from Harlan Sprague Dawley Inc. (Horst, The Netherlands) and were maintained in our central animal facility. The Animal Care and Use Committee of the University of Amsterdam, The Netherlands, approved all experiments.

\subsection*{Production of recombinant AAV}
The rAAV5 constructs were derived from AAV2 and driven by the cytomegalovirus immediate early (CMV) promoter. Recombinant AAV (rAAV) was produced by co-transfection of 293 HEK cells with 150 μg packaging plasmid pDP5 (17) and 50 μg vector plasmid containing the IKKβdn (8) or eGFP gene by the calcium phosphate method. Plasmids were purified with Qiafilter plasmid Giga Kits (Qiagen, Alameda, CA). The packaging plasmids contained all trans-acting elements: Cap-genes, Rep-genes, and adenoviral helper genes. The vector plasmid contained all cis-acting elements: inverted terminal repeats (ITRs), transgene, polyA signal and CMV promoter. Cells were seeded at a density of 3x10^4 cells/cm^2 four days prior to transfection in 850 cm^2 roller bottles and were grown in 50 ml Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA) with glutamax-I (Invitrogen), 10% (v/v) fetal bovine serum (FBS), 60 U/ml penicillin/streptomycin (PS) (Invitrogen) at 37°C. Before transfection, medium was replaced with Iscove’s Modified Dulbecco’s Medium (IMDM; Invitrogen) and at 16h post-transfection the medium was replaced with 50 ml of fresh DMEM/FBS/PS. At 72 h post-transfection, the cells were harvested in 10 ml 50 mM Tris·HCl pH 8.5, 150 mM NaCl, 1 mM MgCl_2, 0.1% (v/v) Triton X-100. Finally, benzonase (Merck, Whitehouse Station, NJ) was added to the lysate to a final concentration of 75 U/ml. Crude cell lysates were further purified with iodixanol gradients (18). Iodixanol step gradients were made in Beckman Quick-seal tubes (25x89 mm; Beckman, Fullerton, CA) and centrifuged for 1 hour at 69,000 rpm 16°C in a type 70Ti rotor (Beckman). Following centrifugation, the 40% iodixanol gradient layer was extracted, diluted 10 times with PBS containing 1 mM MgCl_2 and 2.5 mM KCl, and concentrated to approximately 2 ml with centrificon devices (YM-100; Millipore Corporation, Billerica, MA). Stock viral titers ranging between 10^{11}-10^{12} genomic copies (GC)/ml in saline were reached.

\subsection*{Evaluation of AAV5.IKKβdn mediated NF-κB inhibition in vitro}
Human RA fibroblast-like synoviocytes (FLS), isolated from synovial biopsies as described previously (19), were grown in DMEM/10% FCS and used from passage 3 to 8. For stimulation experiments FLS were seeded into 24-well plates (Costar, Cambridge, MA) at 1x10^4/well,
incubated with either AAV5.IKKβdn or AAV5.eGFP at an MOI of 1000. After 3 days pre-incubation GFP expression was evaluated before cells were stimulated with IL-1β (2.5 ng/ml; R&D Systems, Abingdon, UK). After 30 min stimulation, cells were washed twice with ice-cold PBS to remove all serum proteins and then lysed in 1x SDS-PAGE sample buffer. Total intra-cellular protein was separated by SDS-PAGE on a 10% gel, using Rainbow-colored protein molecular weight markers (Amersham, Little Chalfont, U.K.) as a reference, and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in TBS containing 2% non-fat dry milk (Bio-Rad, Hercules, CA), Na₂VO₄ (2mM) and 0.05% Tween 20 during 1 h. Detection of phosphorylated (ph) and unphosphorylated IκBα was performed by incubating the membranes with monoclonal antibodies (mAbs) to these proteins (Cell Signaling Technology, Beverly, MA) overnight at 4°C. The membranes were subsequently washed and incubated with HRP-labeled secondary Ab (Bio-Rad) in TBS containing 2% non-fat dry milk, Na₂VO₄ (2mM) and 0.05% Tween 20 for 1 h at room temperature, and after extensive washing assayed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK). Densitometry was performed using Quantity One software (Bio-Rad). For evaluating the effect of AAV5.IKKβdn on macrophage cytokine production in vitro, monocytes were isolated from peripheral blood of healthy controls as described previously (20) and allowed to adhere to tissue culture plastic (24 well plates, Costar) for 1 h (1 x 10⁶ cells; 1 ml). Subsequently, non-adherent cells were washed away and cells were cultured for 8-9 days in IMDM containing 10% FCS to obtain macrophages, with half of the medium refreshed every 3 days. Macrophages were incubated with AAV5.IKKβdn or AAV5.eGFP at an MOI of 1000 and typically 3-4 days later GFP expression was observed and cells were stimulated for 24h with IL-1β (2.5 ng/ml; R&D Systems). Supernatants were harvested and the levels of TNFα were determined by sandwich ELISA as described previously (20).

Intra-articular gene therapy in rats with adjuvant arthritis

All rats were immunized at the base of the tail with 1 mg of heat-killed Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) in 0.1 ml mineral oil on day 0 (8). Clinical signs of arthritis (paw swelling) were usually observed by day 10. For i.a. gene transfer the right ankle joints were injected at day 6 (before clinical signs of arthritis) or day 10 (at the onset of clinical symptoms) after immunization in animals anesthetized with isoflurane. The skin was prepared with ethanol and AAV5.IKKβdn or AAV5.GFP (2.5x10¹⁰ GC) were injected anterolaterally into the right ankle joint in a total volume of 50 μl saline using a 31-gauge needle on a glass syringe (21). The course of arthritis was monitored every other day by water displacement plethysmometry. Rats were sacrificed 17 days after intra-articular injection by CO₂ inhalation and hind paws were collected.

Immunohistochemical analysis of synovial cytokine, MMP and TIMP expression

Hind paws were fixed in 4% paraformaldehyde. After 24 h paraformaldehyde was replaced by 70% ethanol and X-rays of the ankle joints were made. Subsequently, the paws were de-
calcified in decalcifying solution (15% EDTA; pH 7.5) on a rotator at 4°C, with the solution changed twice a week. After one week of decalcification the paws were longitudinally cut in half and after four weeks the decalcified paws were dehydrated in graded alcohol and embedded in paraffin. Paraffin-embedded paws were serially sectioned at 4μm thickness. Sections were subsequently deparaffinized in xylene and rehydrated in ethanol, followed by incubation with hydrogen peroxide 30% in 0.1% Na-azide-PBS to block endogenous peroxidase activity. Antigen retrieval was performed by boiling the sections in citrate buffer (pH 6.0) for 10 min. Cytokine expression was studied by staining the sections overnight at 4°C with polyclonal goat antibodies specific for rat TNFα (10 μg/ml), IL-6 (10 μg/ml), IL-10 (10 μg/ml), TIMP-1 (5 μg/ml) (all from R&D Systems) or a mouse monoclonal specific for MMP3 (10 μg/ml) (Chemicon, Temecula, CA) in PBS/BSA 1%. Sections were then washed extensively and incubated with secondary HRP-conjugated swine-anti-goat or goat-anti-mouse antibodies (Dako, Glostrup, Denmark) in PBS/BSA 1% + 10% NHS. Signal amplification was performed using biotinylated tyramine (Perkin Elmer Life Sciences) followed by streptavidine-HRP (Dako-cytomation) in PBS/BSA 1% as described previously (22). Finally, peroxidase activity was detected with AEC (0.02% 3-amino-9-ethylcarbazole; Vector Laboratories, Peterborough, UK) yielding red coloration. Sections were counterstained with Mayer’s haemalum solution (Merck, Darmstadt, Germany) and mounted with Kaiser’s glycerol gelatin (Merck) mounting medium. For quantification of cytokine expression, the sections were analyzed in a random order by computer assisted image analysis (23).

Digital image analysis

Six randomly selected fields within each section were chosen for digitizing the amount of positive signal. These images were acquired on an Olympus microscope (Olympus, Tokyo, Japan), captured using a Charged Coupled Device video camera (Sony, Tokyo, Japan) and digitized with a PV100 multimedia 16-bit color video digitizer card. In the resultant color images the area of positive staining and the mean optical density (MOD) were measured by a macro program as described earlier (24). The MOD is proportional to the cellular concentration of protein. The integrated optical density (IOD) is equal to the MOD multiplied by the area of positive staining.

Culture of synovial biopsies from RA patients and measurement of IL-6 production

Small-bore arthroscopy (2.7mm arthroscope, Storz, Tuttinglen, Germany) was performed under local anesthesia in patients with established RA (25). The obtained biopsies were cultured intact in 1 ml DMEM/ 10% fetal calf serum (FCS) in a humidified 5% CO2 atmosphere in the presence or absence of AAV5.IKKβdn or AAV5.eGFP (2.5x1010 GC/ml). After 3 days biopsies were snap frozen in Tissue-Tek OCT compound (Miles Scientific, Elkhart, IN) and stored at -80°C until sectioning. Serial cryosections were cut and GFP expression was evaluated by immunohistochemical staining using mouse monoclonal anti-GFP JL-8 (2 μg/ml; Clontech, Palo Alto, CA).
Alto, CA), as described previously (26). Transduced biopsies that remained in culture were subsequently stimulated with TNFα (10 ng/ml; R&D Systems). Four days later supernatants were collected and assayed for the presence of IL-6 by sandwich ELISA as described previously (20).

Statistical analysis
Treatment effects in the animal experiments were analyzed using repeated measures ANOVA with treatment and time as fixed factors and rat number as random factor. To test whether treatment induced amelioration of arthritis in time was significant the interaction-test treatment*time was applied (SPSS 11.5.1 Statistics UK). Data from in vitro and ex vivo experiments were analyzed for statistical significance (GraphPad, InStat, version 2.02) using the Student’s t test or Mann-Whitney U test. A p value <0.05 was taken as the level of significance.

RESULTS

Transduction of human FLS with AAV5.IKKβdn blocks IL-1β induced IkBα phosphorylation
To evaluate the effects of AAV5.IKKβdn on cell types that contribute significantly to synovial inflammation, we analyzed the effects of AAV5.IKKβdn transduction on IL-1β stimulation induced NF-κB activation in FLS in vitro. As a readout for NF-κB activation, IKK mediated phosphorylation of IkBα was used. FLS were transduced with AAV5.IKKβdn or control vector containing eGFP and 3 days later GFP expression was evaluated by fluorescent microscopy (Figure 1A). FLS transduction efficiencies were typically around 40-45%, as described earlier (15). Subsequently, cells were stimulated with IL-1β, lysed and total intracellular protein was used for immunoblotting. Phosphorylated (ph) and unphosphorylated IkBα was detected using specific monoclonal antibodies. AAV5.IKKβdn pretreatment resulted in an almost 50% reduction of IL-1β induced IkBα phosphorylation (phIkBα/ IkBα ratio 0.69 ± 0.01 vs. 1.33 ± 0.04 in untransduced control cells or 1.99 ± 0.32 in AAV5.eGFP transduced cells; p<0.05 and p<0.01, respectively). The control vector containing eGFP did not affect IL-1β induced IkBα phosphorylation (phIkBα/ IkBα ratio 1.99 ± 0.32 vs. 1.33 ± 0.04 in control cells; p=ns) (Figure 1B).

Transduction of human macrophages with AAV5.IKKβdn inhibits TNFα production
Macrophages are key players in the ongoing synovial inflammation in RA. Therefore, we investigated the effects of AAV5.IKKβdn on TNFα production by macrophages. Macrophages were transduced with AAV5.IKKβdn 3 days prior to IL-1β stimulation and TNFα in the supernatant was measured after 24h. Although transduction efficiencies were relatively low, AAV5.eGFP was capable to transduce human macrophages in vitro (Figure 1C). Transduction with AAV5. IKKβdn resulted in significantly reduced TNFα production compared to macrophages trans-
duced with AAV5.eGFP (0.3±0.022 vs.0.436±0.018 ng/ml; p<0.05) (Figure 1D). Taken together, we demonstrated that AAV5.IKKβdn blocks IL-1β induced IκBα phosphorylation in FLS and TNFα production in macrophages. Hence, AAV5.IKKβdn is able to transduce and inhibit inflammatory responses in two important cell types involved in the pathogenesis of RA.

Transduction of the synovium is more effective in actively inflamed joints

Next, we investigated the transduction efficiency of synovial tissue by rAAV5 at two different time-points in the disease course of rat AA, in order to establish the optimal time-point for the local treatment of arthritis. Because expression of the transgene requires at least 3-4 days when AAV is used as vector for gene therapy, we injected the animals i.a. at day 6 (before the onset of arthritis) aiming for transgene expression exactly at the onset of clinical signs of
arthritis, or at day 10 (early arthritis). Two weeks after injection we observed a slightly lower overall transduction of the synovial tissue when the vector is injected on day 6, in comparison to injection on day 10 after AA induction (Figure 2A).

Intra-articular AAV5.IKKβdn gene therapy ameliorates active arthritis in rats

Next, we investigated the therapeutic potential of AAV5.IKKβdn for the local treatment of arthritis in rat AA. Because synovial expression of the transgene was only marginally higher after treatment on day 10, we injected the animals i.a. both at day 6 (pre-clinical synovitis) and on day 10 (early arthritis). The course of arthritis was monitored until day 17 after injection to evaluate the effects of intra-articular IKKβ inhibition on paw swelling. Intra-articular treatment with AAV5.IKKβdn on day 6 did not result in delayed onset of arthritis or reduced
Figure 3. Reduced pro-inflammatory cytokine expression in AAV5.IKKβdn treated rats. See Color figures
(A) Expression of pro-inflammatory cytokines in arthritic ankle joints of rats treated with AAV5.IKKβdn on day 10. Representative pictures (overview + detailed image) of synovial cytokine staining are shown, followed by results from digital image analysis of the immunohistochemical stainings (n=10/group; mean ± SEM). TNFα and IL-6 expression was significantly reduced in the AAV5.IKKβdn treated group (p =0.03 and p =0.005 respectively). IL-10 expression was not altered by AAV5.IKKβdn treatment. (B) Expression of pro-inflammatory cytokines in arthritic ankle joints of rats treated with AAV5.IKKβdn on day 6. Results from digital image analysis of immunohistochemical stainings are shown (n=10/group; mean ± SEM). No significant differences in TNFα, IL-6 and IL-10 expression were observed between the groups.
paw swelling (Figure 2B), which could be explained by less efficient transduction of clinically uninvolved joints. Of interest, i.a. treatment of rats at the onset of arthritis (day 10) did result in a significant reduction of arthritis activity \( (p<0.05; \text{AAV5.IKKβdn vs. AAV5.eGFP}) \) starting 5-6 days after i.a. injection (Figure 2C). No contralateral effect was observed. These findings indicate that local AAV5 mediated IKKβ inhibition ameliorates established arthritis.

Reduced synovial inflammation in AAV5.IKKβdn treated rats

Having shown the beneficial effect of AAV5.IKKβdn gene therapy of actively inflamed joints, we evaluated the effects of i.a. AAV5.IKKβdn treatment at day 10 or day 6 on synovial cellularity \textit{in situ}. Intra-articular injection of AAV5.IKKβdn on day 10 resulted in a significant decrease of inflammatory cells in the synovial tissue compared with AAV5.eGFP treatment \((169 \pm 21 \text{ cells/mm}^2 \text{ vs. } 225 \pm 15 \text{ cells/mm}^2, \text{respectively}; p=0.03)\), whereas i.a. AAV5.IKKβdn treatment

Figure 4. Intra-articular injection of AAV5.IKKβdn does not result in reduced bone erosion.

(A) X-rays of the ankle joints were made and radiological damage was scored on a scale of 0-6. Radiological damage was not significantly reduced in the AAV5.IKKβdn treated rats at both time points. (B) Expression of MMP3 in arthritic ankle joints of AAV5.IKKβdn treated early arthritic rats. Results from digital image analysis of immunohistochemical stainings are shown \( (n=10/\text{group}; \text{mean} \pm \text{SEM}) \). No significant difference in MMP3 expression was observed between the two treatment groups. (C) Expression of TIMP-1 in ankle joints of AAV5.IKKβdn treated early arthritic rats. Results from digital image analysis of immunohistochemical stainings are shown \( (n=10/\text{group}; \text{mean} \pm \text{SEM}) \). No significant difference in TIMP-1 expression was observed between the two treatment groups.
on day 6 did not result in significant changes in synovial cellularity (230 ± 12 cells/mm² vs. 223 ± 7 cells/mm², AAV5.IKKβdn vs. AAV5.eGFP respectively; p=ns) (Table 1). Next, we evaluated the effects of i.a. AAV5.IKKβdn treatment at day 10 on synovial cytokine expression in situ. Immunohistochemical analysis of the synovial tissue revealed a clear difference between AAV5.IKKβdn and AAV5.eGFP treated animals in the expression of the pro-inflammatory cytokines TNFα (2765 ± 508 vs. 4547 ± 566; p=0.03) and IL-6 (11461 ± 1521 vs. 20751 ± 2475; p=0.005). IL-10 expression was not different between the two groups (7165 ± 473 vs. 8824 ± 741; p=ns) (Figure 3A). Intra-articular injection of AAV5.IKKβdn on day 6 did not result in significant changes in the expression of these cytokines in synovial tissue (Figure 3B). AAV5.IKKβdn treatment did not reduce bone degradation as quantified by scoring radiographs of the ankle joints (Figure 4A), consistent with previous observations (8). Accordingly, there were no significant differences in the expression of MMP3 (Figure 4B) and TIMP-1 (Figure 4C) in the synovial tissue of AAV5.IKKβdn treated rats on day 10 compared with controls.

AAV5.IKKβdn inhibits TNFα induced IL-6 production in whole tissue synovial biopsies from RA patients

Having demonstrated the anti-inflammatory effects of blocking IKKβ in an animal model of arthritis, we conducted translational experiments in which we evaluated the effects of specific AAV5 mediated IKKβ inhibition on human synovial tissue. Therefore, we obtained synovial biopsies from RA patients by arthroscopy and transduced these with AAV5.IKKβdn or AAV5.eGFP. To assess transduction of these biopsies by AAV5, serial cryosections were cut and GFP expression was detected using immunohistochemistry (because of auto-fluorescence of the synovial tissue). AAV5.eGFP was demonstrated to efficiently transduce human RA synovial biopsies in vitro (Figure 5A). Subsequently, transduced whole tissue biopsies were stimulated with TNFα and IL-6 production was measured by ELISA. AAV5.IKKβdn treatment resulted in a significant reduction of TNFα induced IL-6 production compared to AAV5.eGFP (1.46 ± 0.16 vs. 1.96 ± 0.08; p=0.03) (Figure 5B). These results indicate that AAV5.IKKβdn, in addition to its therapeutic effects in an animal model of arthritis, may also reduce inflammation in synovial tissue from RA patients.
DISCUSSION

In the present study we showed that rAAV5 mediated gene therapy targeting the NF-κB activating kinase IKKβ locally in the joint significantly reduced established arthritis in vivo. In contrast, i.a. injection in a preclinical phase of the disease did not result in amelioration or prevention of arthritis in rats. In addition, we showed that rAAV5 can also be used to target NF-κB in human macrophages and RA FLS. Finally, we demonstrated that rAAV5 transduces intact human RA synovial tissue ex vivo, resulting in reduced TNFα-induced IL-6 production when AAV5.IKKβdn was used to inhibit NF-κB.
Several gene therapeutic approaches for arthritis using rAAV have been successful in animal models of arthritis, both systemically and locally, as introduction of genes encoding cytokines like IL-4 and IL-10 or soluble factors like TNF receptors and IL-1-Ra have proven to reduce the severity of arthritis (reviewed in (27)). However, intracellular signal transduction intermediates have not been targeted before in arthritis using rAAV. Also, in the fore mentioned studies rAAV2 was used as a vector to introduce the transgene. Recently, we demonstrated that rAAV5 is the optimal rAAV vector for intra-articular gene therapy (15,16). Therefore, we tested the capacity of rAAV5 to mediate NF-κB inhibition in arthritis via introduction of the IKKβdn gene, identical to the transgene used in the previously published study using an adenoviral vector (8). rAAV5 mediated transgene expression has several advantages over using an adenoviral vector. Because rAAV contains minimal sequences encoding viral proteins, the induction of an immune response to viral proteins that are co-expressed with the transgene is nearly absent following rAAV transduction (13). Therefore, rAAV results in stable, longterm expression of the transgene (14,15). In addition, neutralizing antibodies against AAV are less prevalent in the general human population allowing better transduction (28). In this respect, usage of rAAV5 as a vector also has an advantage over rAAV2, as literature suggests that neutralizing antibodies to AAV2 are more prevalent than neutralizing antibodies to AAV5 and the presence of neutralizing antibodies to AAV2 does not prevent efficient transduction with rAAV5 (15,29). We also observed less neutralizing antibody formation in the serum of rats that were treated with rAAV5 compared to rAAV2 treated rats (15). This may have important implications for baseline transduction efficiency, re-administration purposes or treatment of multiple joints at different time-points.

In the current study we first demonstrated that AAV5.IKKβdn was able to suppress inflammatory properties of two important cell types in the pathogenesis of RA: human macrophages and RA FLS in vitro. After these validation studies we tested the vector in rat AA and showed that AAV5.IKKβdn significantly reduced the severity of arthritis. This was accompanied by a decreased synovial cellularity and resulted in a reduction of the proinflammatory cytokines TNFα and IL-6 in the synovial tissue, whereas expression of the anti-inflammatory cytokine IL-10 was unaffected. These findings suggest that AAV5.IKKβdn selectively targets NF-κB responsive pro-inflammatory cytokines, while leaving beneficial mediators like IL-10 and TIMP-1 unaffected, thereby shifting the balance to a more anti-inflammatory state resulting in reduced synovial inflammation.

AAV5.IKKβdn treatment did not reduce bone destruction, and in line with this we observed no effect on synovial MMP3 expression. A possible explanation is the fact that MMP expression is not only NF-κB responsive, but also to a large extent dependent on the transcription factor activator protein (AP)-1 (2). Although regulated cross-talk exists between the AP-1 and NF-κB pathways, as it has been demonstrated that IKKβ plays an important role in the tran-
scription of \textit{junB}, \textit{c-fos}, \textit{fosB} and \textit{fra-2} genes that encode essential components of AP-1 (30), AP-1 activation is thought to peak earlier than NF-κB in arthritis and could therefore be unaffected by AAV5.IKKβdn treatment in our study. Because rAAV mediated gene therapy results in transgene expression not earlier than 3-4 days after i.a. injection of the vector (i.e. on day 13-14 after arthritis induction), IKKβ inhibition likely occurred too late to alter joint destruction. However, MMPs are not the only important mediators of bone erosion, as osteoclasts are also involved in this process. In our hands preliminary experiments to transduce human osteoclasts with rAAV5 were not successful \textit{in vitro} (data not shown). This may to some extent explain the lack of an osteoprotective effect in our study, whereas other studies targeting NF-κB locally in the joint, for example using the small molecule inhibitor NBD peptide that is able to enter most cell types in the joint, did result in reduced bone erosion (9) (Chapter 6).

Another important finding of our study is that i.a. injection of the vector in a pre-clinical phase of the disease did not result in significant amelioration of the arthritis. This was partly due to less overall synovial transduction, but could potentially also be due to transduction of different cell types by rAAV5 in different phases of the disease, which is likely to result in differential therapeutic effects. Also, the nature of the transgene may in part account for this, as we introduced a dominant negative signal transduction intermediate. This should exert its effect in all inflammatory cells, whereas introduction of a gene encoding a secretory therapeutic protein like anti-TNFα preferentially requires transduction of a stable cell population in the joint like FLS, to ensure continuous production.

Although rAAV generally transduces cells less efficiently than an adenoviral vector expressing the same transgene or small molecule inhibitors, the stable longterm expression of the transgene makes it an attractive candidate for treating chronic inflammatory diseases like RA (27,31). In addition, we showed that adenoviral vectors almost exclusively transduce the synovial lining layer, whereas AAV vectors penetrate much further into the synovium, thereby transducing also the synovial sublining (15).

Our results indicate that AAV5.IKKβdn may have therapeutic potential in humans as well, because TNFα induced pro-inflammatory cytokine production in cultured synovial biopsies from RA patients was efficiently blocked. In conclusion, our results demonstrate that AAV5 mediated gene therapy targeting NF-κB locally in the joint effectively suppresses synovial inflammation and may be a future treatment for RA patients with recurring inflammation in one or more joints despite optimal systemic treatment.


Chapter 6

Local treatment with the selective \( \text{IkB} \) kinase \( \beta \) inhibitor NEMO binding domain peptide ameliorates synovial inflammation

Sander W. Tas\(^1\), Margriet J. Vervoordeldonk\(^1\), Najat Hajji\(^1\), Michael J. May\(^2\), Sankar Ghosh\(^3\), and Paul P. Tak\(^1\)

\(^1\)Div. of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, The Netherlands. \(^2\)School of Veterinary Medicine, Dept. of Animal Biology, University of Pennsylvania, Philadelphia, \(^3\)Immunobiology Section, Yale University Medical School, New Haven, CT.
ABSTRACT

Nuclear factor (NF)-κB is a key regulator of synovial inflammation. We investigated the effect of local NF-κB inhibition in rat adjuvant arthritis (AA), using the specific IKKβ blocking NEMO binding domain (NBD) peptide. The effects of the NBD peptide on human fibroblast-like synoviocytes (FLS) and macrophages, as well as rheumatoid arthritis (RA) whole tissue biopsies were also evaluated. First, we investigated the effects of the NBD peptide on RA FLS in vitro. Subsequently, NBD peptides were administered intra-articularly (i.a.) into the right ankle joint of rats at the onset of disease. The severity of arthritis was monitored over time, rats were sacrificed on day 20 and tissue specimens were collected for routine histology and X-rays of the ankle joints. Human macrophages or RA synovial tissue were cultured ex vivo in the presence or absence of NBD peptides and cytokine production was measured in the supernatant by ELISA. The NBD peptide blocked IL-1β-induced IkBα phosphorylation and IL-6 production in RA FLS. Intra-articular injection of the NBD peptide led to significantly reduced severity of arthritis (p<0.0001) and radiological damage (p=0.04). This was associated with decreased synovial cellularity and reduced expression of TNFα and IL-1β in the synovium. Incubation of human macrophages with NBD peptides resulted in 50% inhibition of IL-1β-induced TNFα production in the supernatant (p<0.01). In addition, the NBD peptide decreased TNFα-induced IL-6 production by human RA synovial tissue biopsies by approximately 42% (p<0.01). Specific NF-κB blockade using a small peptide inhibitor of IKKβ has anti-inflammatory effects in AA and human RA synovial tissue, as well as in two important cell types in the pathogenesis of RA: macrophages and FLS. These results indicate that IKKβ targeted NF-κB blockade using the NBD peptide could offer a new approach for the local treatment of arthritis.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting predominantly the joints (1). Many different cell types have been described to contribute to both the initiation phase of the disease and the chronic perpetuation of synovial inflammation. In rheumatoid synovium the intimal lining layer shows marked hyperplasia, mainly due to expansion of intimal macrophages and fibroblast-like synoviocytes (FLS) (2). Macrophages appear to play a pivotal role in the pathogenesis of RA as they are present in high numbers in RA synovial tissue and clearly show signs of activation including enhanced expression of cellular surface markers like MHC class II molecules, pro-inflammatory cytokines like tumor necrosis factor α (TNFα) (3), chemokines, and matrix metalloproteinases (MMPs) (4). Furthermore, there is a highly significant positive correlation between scores for local disease activity and macrophage numbers and the expression of macrophage-derived cytokines in the synovium (5). In addition to macrophages, other cell types, like FLS, also display altered biology. RA FLS are characterized by anchorage-independent growth and resistance to apoptosis due to constitutive activation of multiple signaling cascades (reviewed in (6,7)).

In many of the cells involved in synovial inflammation alterations are found in intracellular signaling cascades leading to unwanted interactions with other cells resulting in pathology (8). Striking abnormalities are observed in the NF-κB signal transduction pathway (9). Phosphorylation of inhibitor of κB (IκBα) by the IκB kinase (IKK) complex is a crucial step in NF-κB/Rel activation. The IKK complex contains two catalytic subunits, named IKKα and IKKβ, and a regulatory subunit termed NEMO (NF-κB essential modulator). NF-κB activation in response to pro-inflammatory signals is mainly dependent on IKKβ (10). The subsequent polyubiquitination targets IκBα for degradation, releasing NF-κB dimers from the NF-κB-IκBα complex, followed by translocation to the nucleus and binding to κB enhancer elements of target genes (11).

IKK is a key convergence site of many different stimuli that induce NF-κB activation such as pro-inflammatory cytokines and ligation of Toll-like receptors, but triggering of highly specialized antigen receptors such as the T cell receptor is also dependent on this pathway (12). Consequently, selective inhibition of the IKK complex has emerged as a promising strategy to block aberrant NF-κB activity in autoimmune and inflammatory diseases as well as certain cancers (13).

NF-κB is highly activated in synovial tissue of RA patients (14,15), with IKKβ being a key regulator of synovial inflammation (16). Various local or systemic approaches to specifically inhibit the activation of this transcription factor by targeting the IKK complex have proven successful in the amelioration of arthritis (16-19). Obviously, NF-κB activity is also required for normal
physiology of cells or for clearing microbial pathogens, raising toxicity concerns when this pathway is blocked systemically in many different cell types at the same time. Accordingly, for development of therapies blocking NF-κB activity in RA, local intra-articular therapy appears more attractive.

The present study was conducted to explore the effects of specific inhibition of IKKβ-mediated NF-κB activation locally in the inflamed joint using the well-characterized NEMO-binding domain (NBD) peptide (20). Our data indicate that local IKKβ targeted NF-κB blockade using a small peptide inhibitor ameliorates synovial inflammation, both in an animal model of arthritis and in human RA synovial tissue ex vivo, which opens up a new approach for the local treatment of RA.

**MATERIAL AND METHODS**

**Animals**

Pathogen-free male Lewis rats (150-200 g; 8-10 weeks of age at the start of the experiments) were obtained from Harlan Sprague Dawley Inc. (Horst, The Netherlands) and were maintained in our central animal facility. The Animal Care and Use Committee of the University of Amsterdam, The Netherlands, approved all experiments.

**NBD peptides**

Small scale Fmoc synthesis of the peptides was carried out on a Rainin Symphony Instrument at the HHMI Biopolymer-Keck Foundation Biotechnology Resource Laboratory at Yale University. Peptides were characterized by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and analytical reverse phase HPLC analysis. The peptides were subsequently dissolved in DMSO to a stock of 50 mM. The sequences of the wild-type and mutant (MUT) NBD peptides have been described previously (20). The NBD peptide (3.7 kD) contains the region of IKKβ from T735 to E745 synthesized in tandem with a membrane permeabilization sequence from the drosophila antennapedia homeodomain protein. The MUT peptide (3.5 kD) is identical except that W739 and W741 are replaced by alanines to render it biologically inactive.

**Evaluation of NF-κB inhibition in FLS**

Human RA FLS, isolated from synovial biopsies as described previously (21), were grown in DMEM/10% FCS and used from passage 3 to 8. For stimulation experiments FLS were seeded onto 24 wells dishes (Costar, Cambridge, MA) at 1 x10⁴/well. After serum starving for 12 hours for synchronization, cells were pre-incubated for 2h with NBD or MUT peptides (50 μM) in medium containing 0.5% FCS and stimulated with IL-1β (2.5 ng/ml). After 30 min stimulation,
cells were washed twice with ice-cold PBS to remove all serum proteins and then lysed in 1x SDS-PAGE sample buffer. Total intra-cellular protein was separated by SDS-PAGE on a 10% gel, using Rainbow-colored protein molecular weight markers (Amersham, Little Chalfont, U.K.) as a reference, and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in TBS containing 2% non-fat dry milk (Bio-Rad, Hercules, CA), Na$_3$VO$_4$ (2mM) and 0.05% Tween 20 during 1 h. Detection of phosphorylated and unphosphorylated proteins was performed by incubating the membranes with a primary antibody against the protein of interest overnight at 4°C. The membranes were subsequently washed and incubated with the appropriate HRP-labeled secondary Ab (Bio-Rad) in TBS containing 2% non-fat dry milk, Na$_3$VO$_4$ (2mM) and 0.05% Tween 20 for 1 h at room temperature, and after extensive washing assayed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK). Mouse mAbs to phosphorylated (ph) I$\kappa$B$\alpha$ and total I$\kappa$B$\alpha$ were obtained from Cell Signaling Technology (Beverly, MA). Densitometry was performed using Quantity One software (Bio-Rad). To study the effects of NBD on cytokine production, FLS were pre-incubated for 2h with NBD peptides (50 μM) and stimulated for 24h with rhIL-1β (2.5 ng/ml; R&D Systems). Supernantants were harvested and the levels of IL-6 were determined by sandwich ELISA as described previously (22).

Adjuvant arthritis

All rats were immunized at the base of the tail with 1 mg of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) in 0.1 ml mineral oil on day 0 (16). Clinical signs of arthritis (i.e. paw swelling) were usually observed by day 10 and monitored during the course of disease by water displacement plethysmometry. Paw swelling was expressed as delta paw volume, i.e. with paw volume before onset of arthritis subtracted. For intra-articular treatment the right ankle joints were injected at day 10 and 12 after immunization in animals (n=6/group (dose-finding); n=15/group (clinical study)) anesthetized with isoflurane. The skin was prepared with ethanol and NBD or MUT peptides were injected in the indicated concentrations anterolaterally into the right ankle joint in a total volume of 50 μl saline using a 31-gauge needle on a glass syringe (23). The course of arthritis was monitored regularly until rats were sacrificed at day 20 by CO$_2$ inhalation and hind paws were collected. X-rays of the ankle joints were made and these plain radiographs were scored for bone degradation using a semiquantitative scoring system (demineralization [0-2+], ankle and midfoot erosions [0-2+], calcaneal erosion [0-1+], heterotopic bone formation [0-1+]; maximum possible score = 6) according to Boyle et al. (24).

Immunohistochemical analysis of synovial cytokine expression

Hind paws were obtained from each rat, trimmed of skin, and fixed in 4% paraformaldehyde. After 24 h paraformaldehyde was replaced by 70% ethanol and X-rays of the ankle joints were made. Subsequently, the paws were decalcified for 4 weeks in decalcifying solution (15% EDTA pH 7.5) on a rotator at 4°C, with decalcifying solution changed twice a week. After
one week of decalcification the paws were longitudinally cut in half. After four weeks the formalin-fixed paws were dehydrated in graded alcohol and embedded in paraffin. Paraffin-embedded paws were serially sectioned at 4μm thickness. Sections were subsequently deparaffinized in xylene and rehydrated in ethanol, followed by incubation with hydrogen peroxide 30% in 0.1% Na-azide-PBS to block endogenous peroxidase activity. Antigen retrieval was obtained by boiling the sections in citrate buffer pH 6.0 for 10 min. Cytokine expression was studied by staining the sections overnight at 4°C with monoclonal antibodies specific for TNFα (10 μg/ml), IL-6 (10 μg/ml) and IL-1β (10 μg/ml) (all from R&D Systems, Oxon, UK) in PBS/BSA 1%. Sections were then washed extensively and incubated with secondary HRP-conjugated swine-anti-goat antibodies (Dako, Glostrup, Denmark) in PBS/BSA 1% + 10% NHS. Signal amplification was performed using biotinylated tyramine (Perkin Elmer Life Sciences) followed by streptavidine-HRP (Dakocytomation) in PBS/BSA 1% as described previously (4). Finally, peroxidase activity was detected with AEC (0.02% 3-amino-9-ethylcarbazole, Vector Laboratories) yielding red coloration. Sections were counterstained with Mayer’s haemalum solution (Merck, Darmstadt, Germany) and mounted with Kaiser’s glycerol gelatin (Merck) mounting medium. For quantification of cytokine expression, the sections were blinded and analyzed in a random order by computer assisted image analysis.

Digital image analysis
Six randomly selected fields within each section were chosen for digitizing the amount of positive signal. These images were acquired on an Olympus microscope (Olympus, Tokyo, Japan), captured using a Charged Coupled Device video camera (Sony, Tokyo, Japan) and digitized with a PV100 multimedia 16 bit color video digitizer card. In the resultant color images the area of positive staining and the mean optical density (MOD) were measured by a macro program as described earlier (25,26). The MOD is proportional to the cellular concentration of protein. The integrated optical density (IOD) is equal to the MOD multiplied by the area of positive staining.

Culture of normal macrophages and synovial biopsies from RA patients
For evaluating the effect of NBD on macrophage cytokine production in vitro, monocytes were isolated from peripheral blood of healthy controls as described previously (22) and allowed to adhere to tissue culture plastic (24 well plates; Costar) for 1h (1 x 10⁶ cells; 1 ml). Subsequently, non-adherent cells were washed away and cells were cultured for 8-9 days in IMDM containing 10% FCS to obtain macrophages, with half of the medium refreshed every 3 days. Macrophages were pre-incubated for 2h with NBD peptides (50 μM) and cells were stimulated for 24h with rhIL-1β (2.5 ng/ml; R&D Systems). Supernantants were harvested and the levels of TNFα were determined by sandwich ELISA as described previously (22). To evaluate the effects of NBD on human synovial tissue, small-bore arthroscopy (2.7mm arthroscope, Storz, Tuttlingen, Germany) was performed under local anesthesia in patients
Local treatment with the NBD peptide ameliorates synovial inflammation with established RA (27). The obtained biopsies were cultured intact in DMEM/10% fetal calf serum (FCS) in a humidified 5% CO₂ atmosphere in the presence or absence of NBD peptides (100 μM) and after 2h pre-incubation stimulated with rhTNFα (10 ng/ml; R&D Systems). After 7 days supernatants were collected and evaluated for the presence of IL-6 by sandwich ELISA as described previously (22). IL-6 levels were corrected for size of the biopsies by weighing of the biopsies at day 7.

Statistical analysis
Treatment effects in the animal experiments were analyzed using repeated measures ANOVA with treatment and time as fixed factors and rat number as random factor. To test whether treatment induced amelioration of arthritis in time was significant the interaction-test treatment*time was applied (SPSS 11.5.1 Statistics UK), resulting in the “area-under-the-curve” (AUC). Data from in vitro and ex vivo experiments were analyzed for statistical significance (GraphPad, InStat, version 2.02) using the Student’s t test or Mann-Whitney U test. A p value <0.05 was taken as the level of significance.

RESULTS

NBD peptide blocks IκBα phosphorylation and IL-6 production in RA FLS
The NF-κB blocking effect of the NBD peptide has been extensively characterized (17,18,20,28). To evaluate the effects of IKKβ inhibition in RA FLS, we analyzed the consequences of pretreatment with NBD on IL-1β induced IKK mediated phosphorylation of IκBα as readout for NF-κB activation in vitro. FLS were pre-treated for 2h with NBD or the mutant control peptide and stimulated for 30 min with IL-1β. Cells were lysed and total intracellular protein was separated using SDS PAGE gel electrophoresis. After immunoblotting, phosphorylated and unphosphorylated IκBα were detected using specific monoclonal antibodies. NBD pretreatment resulted in reduced IL-1β-induced IκBα phosphorylation, whereas the mutant control peptide did not affect IκBα phosphorylation in FLS. Using densitometry we found that the phospho-IκBα/IκBα ratio was significantly reduced in NBD compared to MUT treated FLS (P < 0.01). NBD treatment almost reduced the ph IκBα/IκBα ratio to the level seen in unstimulated cells (0.47 vs. 0.21 in unstimulated cells). The mutant control peptide (MUT) did not affect IκBα phosphorylation (ph IκBα/IκBα ratio 1.20 vs.1.13 in stimulated control cells) (Figure 1A). The low ph IκBα/IκBα ratio after NBD treatment of FLS was accompanied by a strong reduction in IL-1β-induced IL-6 secretion by these cells (1.4 ± 0.1 ng/ml vs. 8.5 ± 1.3 ng/ml in stimulated cells and 14.3 ± 4 ng/ml in MUT treated stimulated FLS, P < 0.01) (Figure 1B). Taken together, we demonstrate that the NBD peptide blocks IL-1β induced IκBα phosphorylation in RA FLS resulting in a less inflammatory phenotype.
Intra-articular NBD treatment ameliorates adjuvant arthritis in rats

Next we investigated the therapeutic effects of this highly specific IKKβ inhibitor in established arthritis when administered intra-articularly. In a dose finding study (n=6/group) we found that two i.a. injections (on day 10 and 12) with a dose of 150 μg NBD peptide significantly reduced arthritis severity (P < 0.05), whereas a dose of 50 μg only marginally ameliorated arthritis (Figure 2A). Subsequently, we conducted a large therapeutic study in which adjuvant arthritis was induced in rats (n=15/group) on day 0. At the start of arthritis symptoms (day 10), the animals received an intra-articular injection with either the NBD peptide or the MUT peptide (150 μg) into the right ankle joints. Two days later this procedure was repeated and the course of arthritis was monitored by blinded observers until day 20 to evaluate the effects of local IKKβ inhibition on paw swelling. Intra-articular treatment with NBD resulted in significantly reduced paw swelling (AUC 11.18 ± 1.14 vs. 15.40 ± 0.70, NBD vs. MUT respectively; P < 0.0001)(Figure 2B+C). In short, we have demonstrated in two independent AA experiments that two i.a. injections of the NBD peptide significantly ameliorated arthritis severity.

Figure 1. NBD peptide blocks IL-1β-induced IkBα phosphorylation and IL-6 production in fibroblast-like synoviocytes (FLS).

(A) FLS were pre-incubated with either NBD or MUT peptide at a concentration of 50 μM for 2 h. Subsequently, cells were stimulated with IL-1β (2.5 ng/ml) for 30 min, extensively washed and lysed in sample buffer. Cell lysates were analyzed by Western blotting. One representative blot out of three is shown. Densitometry includes all three experiments and data are expressed as mean ± SEM (* P < 0.01). (B) NBD peptide blocks IL-1β-induced IL-6 production by FLS in vitro. FLS were pre-incubated with either NBD or MUT peptide at a concentration of 50 μM for 2 h. Subsequently, cells were stimulated with IL-1β (2.5 ng/ml). After 24 h, supernatants were collected and IL-6 levels were measured by sandwich ELISA. Data are representative of three independent experiments performed in triplicates (* P <0.01).
Local treatment with the NBD peptide ameliorates synovial inflammation

Intra-articular NBD treatment results in reduced synovial inflammation

Having shown the beneficial effect of the NBD peptide on the severity of arthritis, we evaluated the effects of i.a. NBD treatment on synovial cellularity in situ. Intra-articular injection of the NBD peptide resulted in a significant decrease of inflammatory cells in the synovial tissue compared with MUT treatment (239 ± 5 cells/mm² vs. 273 ± 7 cells/mm², respectively; P < 0.01). Histological evaluation of NBD treated ankle joints revealed less proliferation and invasive growth of the synovial tissue (Figure 3). Next, we evaluated the effects of the NBD peptide on synovial inflammation by performing immunohistochemical stainings on sections from paraffin embedded rat ankle joints. Digital image analysis of comparable locations in the synovial tissue showed a clear difference between NBD and MUT treated animals in...
the expression of the pro-inflammatory cytokines TNF\(\alpha\) (4.06E+02 ± 2.40E+01 vs. 8.51E+02 ± 2.38E+02; \(P = 0.05\)) and IL-1\(\beta\) (1.73E+04 ± 2.43E+03 vs. 2.66E+04 ± 5.30E+03; \(P = 0.04\)). IL-6 expression was not different between the two groups (1.70E+04 ± 2.17E+03 vs. 1.87E+04 ± 2.10E+03) (Figure 3).

Intra-articular NBD treatment reduces bone destruction

In addition to the effects of NBD treatment on synovial inflammation, we also studied the effects on bone destruction. Therefore, X-rays of the ankle joints (Figure 4A) were made and these plain radiographs were scored for bone degradation using a validated scoring system (24). Intra-articular NBD treatment significantly reduced bone degradation of the injected ankle joints (\(P < 0.04\))(Figure 4B) compared to MUT treated or contralateral joints. These findings show that local IKK\(\beta\) inhibition in the joint by i.a. injection of the small molecule NBD peptide not only ameliorates arthritis, but concomitantly also reduces bone destruction.
Local treatment with the NBD peptide ameliorates synovial inflammation

The NBD peptide inhibits pro-inflammatory cytokine production in human macrophages and whole tissue synovial biopsies from RA patients

To gain more knowledge on the therapeutic potential of the NBD peptide in the human situation, we extended the experiments to another pivotal cell type in the pathogenesis of RA, the macrophage. We found that NBD treatment of human macrophages results in significantly reduced IL-1β induced TNFα production, compared to MUT treated macrophages ($P < 0.01$) (Figure 5A). Finally, we conducted true translational research in which we evaluated the effects of our highly specific IKKβ inhibitor on human synovial tissue. Therefore, we collected synovial biopsies from RA patients by arthroscopy and cultured them in the presence or absence of the NBD peptide, followed by TNFα stimulation. TNFα was chosen for stimulation of whole synovial tissue biopsies as this cytokine has been demonstrated to be pivotal in the pathogenesis of RA (reviewed in (29)). Supernatants were collected and IL-6 production was measured by ELISA. NBD treatment resulted in a significant reduction of TNFα induced IL-6 production compared to MUT treatment or TNFα stimulation alone ($2.99 \pm 0.01$ vs. $5.18 \pm 0.61$)

Figure 4. NBD peptide treatment significantly reduces bone destruction.

(A) X-rays of the ankle joints (n=10/group) were made and radiological damage was scored. Representative pictures for NBD and MUT treated ankle joints are shown. Big arrow indicates ankle demineralization. Small arrow indicates midfoot demineralization and erosions. (B) NBD peptide treatment significantly reduces bone destruction. Data represent mean ± SEM radiological scores (* $P < 0.04$). (C) No significant difference in bone destruction was observed in the contralateral, non-injected paws. Data represent mean ± SEM radiological scores.
Chapter 6

In line with previous observations (17,18), no effect of the NBD peptides on basal IL-6 production was observed (data not shown), as the NBD peptide selectively blocks the induction of NF-κB activity in response to pro-inflammatory stimuli without affecting basal NF-κB activity (20). In conclusion, these experiments demonstrate the effectiveness of the NBD peptide in human cells.

DISCUSSION

In the present study we show for the first time that intra-articular administration of the highly specific IKKβ inhibitor NBD peptide significantly reduces arthritis activity and bone destruction in vivo. These results indicate that IKKβ targeted NF-κB inhibition using selective pharmacological inhibitors is beneficial in the local treatment of established arthritis. Of note, only two i.a. injections with the NBD peptide resulted in sustained reduction of the severity of arthritis in a therapeutic setting. Consistent with these observations, synovial inflammation was decreased as demonstrated by a decline in synovial cellularity and reduced levels of the pro-inflammatory cytokines TNFα and IL-1β. Importantly, intra-articular NBD treatment concomitantly resulted in reduced bone destruction, in agreement with the effects shown after systemic treatment in murine collagen-induced arthritis (18).

Figure 5. NBD peptide blocks pro-inflammatory cytokine production by human macrophages in vitro and RA synovial biopsies ex vivo.

(A) NBD peptide blocks IL-1β-induced TNFα production by human macrophages in vitro. Macrophages were pre-incubated with either NBD or MUT peptide at a concentration of 50 μM for 2 h. Subsequently, cells were stimulated with IL-1β (2.5 ng/ml). After 24 h, supernatants were collected and TNFα levels were measured by sandwich ELISA. Data are representative of three independent experiments performed in triplicates and are expressed as mean ± SEM (* P < 0.01). (B) NBD peptide blocks TNFα-induced IL-6 production of RA synovial biopsies ex vivo. Synovial biopsies were cultured with TNFα (10 ng/ml) in the presence or absence of NBD peptides (100 μM). After 7 days, supernatants were collected and IL-6 levels were measured by sandwich ELISA and corrected for weight of the biopsy. Data are representative of three independent experiments performed in triplicates and are expressed as mean ± SEM (* P < 0.01).
Local treatment with the NBD peptide ameliorates synovial inflammation

The biological effects of local NBD treatment are also consistent with those observed using a gene therapy approach to target IKKβ locally in the joint (16). Selective pharmacological NF-κB inhibitors may reach the clinic faster, because of possible safety and dose regulation issues that accompany gene therapy. However, some of these issues might be resolved by using vectors optimized for intra-articular use like rAAV5 (30) and disease-inducible promoters or other regulatable gene expression systems (31).

In RA, FLS and macrophages play important roles in the perpetuation of synovial inflammation (5,6). Our results indicate that the NBD peptide may have great potential in humans as well, because this NF-κB inhibitor efficiently blocked IL-1β induced IκBα phosphorylation and IL-6 production in RA FLS, as well as TNFα production by human macrophages. One of the important advantages of the NBD peptide, also compared to other IKK inhibitors, is that basal NF-κB activity remains unaffected while NF-κB activation in response to pro-inflammatory stimuli is effectively blocked (20). Therefore, the beneficial role of NF-κB in normal cellular functions is preserved, resulting in less toxicity. Consequently, the effects of the NBD peptide on pro-inflammatory cytokine production in vitro were not due to increased apoptosis (data not shown). In addition, TNFα induced pro-inflammatory cytokine production in cultured synovial biopsies from RA patients was also significantly reduced. In these synovial biopsies the micro-architecture of the synovium is preserved, which allows studying the effects of the NBD peptide on synovial inflammation in the complex, biologically relevant network of cells that contribute to the inflammatory process, rather than in individually cultured cell types. Thus, this may serve as a model to predict a possible therapeutic effect in human disease (32).

Many anti-inflammatory drugs used in the treatment of arthritis target, at least in part, NF-κB and of these drugs glucocorticoids like dexamethasone and prednisolone are considered the most powerful, although non-specific, NF-κB inhibitors (33,34). Intra-articular steroid injections are widely used to control local inflammation. Besides local side-effects such as reduced bone formation (35), recent work has shown unwanted systemic effects due to absorption of steroids from the intra-articular space (36,37). The most common side-effect caused by systemic absorption of intra-articular steroids is suppression of pituitary-adrenal axis function (37). This suppression may last from up to two weeks to even six months after i.a. injection and may ultimately lead to adrenal failure (38). Therefore, there is a clear need for potent anti-inflammatory drugs for intra-articular administration without steroid action to prevent these unwanted side-effects. Pharmacological NF-κB inhibitors like the NBD peptide may fulfill this need as they selectively block NF-κB activity in the joint without causing these side-effects as they are not steroid-, but mainly peptide-based. In addition, the NBD peptide inhibits only pro-inflammatory IKK activity and may therefore be safer than other IKK inhibitors if absorbed from the intra-articular space and released systemically. However, extensive
pharmacological evaluation of this approach is required to carefully monitor pharmacokinetics and –dynamics, as well as potential toxicity of new pharmacological NF-κB inhibitors, like the NBD peptide, before clinical trials with these compounds may be initiated.

In conclusion, we have demonstrated that local small peptide mediated NF-κB inhibition not only ameliorated established arthritis and reduced bone destruction in an animal model of RA, but also prevented pro-inflammatory cytokine production by human RA synovial biopsies. Our results suggest that intra-articular treatment with the NBD peptide may represent a novel therapeutic approach in RA.

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Local treatment with the NBD peptide ameliorates synovial inflammation

REFERENCE LIST


Rheumatoid arthritis synovium contains immature myeloid and plasmacytoid dendritic cells with distinct cytokine profiles

M. Cristina Lebre¹, Sarah L. Jongbloed², Sander W. Tas¹, Petra Reinders-Blankert¹, Tom J.M. Smeets¹, Iain B. McInnes², and Paul P. Tak¹

¹Div. of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. ²Division of Immunology, Infection and Inflammation, Glasgow Royal Infirmary, Glasgow, Scotland.
ABSTRACT

Objective: Dendritic cells (DC) have been proposed to play a pivotal role in the initiation and perpetuation of rheumatoid arthritis (RA) by presentation of arthritogenic antigens to autoreactive T cells. We undertook this study to gain insight into the in vivo characteristics of two major DC subsets, myeloid (m) and plasmacytoid (p)DC, in RA synovial tissue by investigating their frequency, phenotype, distribution and cytokine expression.

Methods: Synovial tissue was obtained from 24 patients with RA, 10 with psoriatic arthritis (PsA) and 12 with inflammatory osteoarthritis (OA). Immunohistochemistry of synovial tissue was performed using specific antibodies against mDC and pDC and stained sections were evaluated by digital image analysis. Coexpression and distribution of mDC and pDC with lineage markers and T cell markers was performed by double immunolabelling. To evaluate the maturation status and the coexpression of cytokines by mDC and pDC, double immunofluorescence microscopy was used and the numbers of double positive DC were quantified.

Results: In all diagnostic groups the number of pDC was significantly higher compared to mDC. In RA synovial tissue both mDC and pDC were localized in lymphocyte aggregates. Analysis of cytokine expression in RA synovial tissue revealed that the percentage of pDC expressing IL-18 and IFN-α was significantly higher, and the percentage of pDC expressing IL-12p70 and IL-23p19 was significantly lower compared to mDC.

Conclusion: Our results indicate that mDC and pDC in synovial tissue may play an important role in synovial inflammation possibly via stimulation of memory T cells. Moreover, the release of proinflammatory and Th1-associated cytokines by DC may sustain the inflammatory process. Conceivably, immunomodulation by targeting synovial tissue DC might provide a novel antirheumatic strategy.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, autoimmune disease that affects approximately 1% of the population (1). The target tissue is primarily the synovial tissue which is characterized by a massive accumulation of leukocytes that comprises mostly T cells, macrophages and plasma cells, but also B cells, dendritic cells (DC) and other cells (2).

Whereas macrophages represent one group of antigen-presenting cells (APC) integral to the pathology of synovial inflammation (2,3), DC, key professional antigen-presenting cells (APC) possessing a unique ability to stimulate naive T cells, represent another (4). There is considerable intra- and inter-tissue variation in the phenotype, morphology, function, and tissue localization of different DC populations (4). Human blood DC have recently been divided into five distinct subsets: CD1b/c+, CD16+, BDCA3+, CD123+ (IL-3R α-chain) and CD34+ DC (5). In particular, the so-called myeloid DC (mDC) which are CD1c (BDCA1)+/CD11c+/CD45RO+/CD123lo have the ability to produce IL-12 in response to bacterial compounds or CD40L, and require GM-CSF for survival (6). Conversely, plasmacytoid DC (pDC) are CD303 (BDCA2)+/CD304(BDCA4)+/CD11c+/CD45RA+/CD123high and require the presence of IL-3 for survival (7,8). Upon viral or bacterial infection or exposure to immune complexes consisting of anti-double stranded DNA, pDC produce high amounts of type I IFNs, IFN-α and IFN-β (9-11).

It has previously been demonstrated that DC infiltration into affected autoimmune tissue occurs early in disease pathology and that DC are enriched in the synovial tissue and synovial fluid of affected joints (12,13). In this respect, DC may play a role in the initiation and perpetuation of RA by presentation of arthritogenic antigen(s) to autoreactive T cells (12,14,15). Moreover, these DC may activate infiltrating T cells and this might be sufficient to drive inflammation and disease. In view of these observations, we propose that synovial tissue DC are not only crucial for (auto)antigen capture leading to autoimmunity and disease initiation, but also have a crucial role in established inflammation. To provide more insight into the role of DC in the pathogenesis of inflammatory joint disease we investigated the frequency, phenotype, distribution and cytokine expression of mDC and pDC subsets present in synovial tissue by immunohistochemical analysis using a panel of specific antibodies.

PATIENTS AND METHODS

Patients and tissue samples
Twenty-two RA patients (16), eleven inflammatory osteoarthritis (OA) patients and ten psoriatic arthritis (PsA) patients were included in this study (Table 1). All patients gave informed consent, and the study protocol was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam. Patients were allowed to use certain disease modifying...
anti-rheumatic drugs (DMARDs) such as methotrexate (no more than 15 mg/week), hydroxychloroquine, or sulfasalazine, provided that the dose had been stable for at least two months. Non-steroidal anti-inflammatory drugs (NSAIDs) were allowed, provided that the dose and frequency had been stable for 30 days. Small-bore arthroscopy was performed under local anesthesia and synovial tissue samples were obtained from multiple sites in the joint using 2-mm grasping forceps (Storz, Tuttlingen, Germany) as previously described (17). Synovial biopsy samples were collected and snap-frozen in TissueTek OCT (Miles, Elkhart, IN). Frozen blocks were stored in liquid nitrogen until sectioned for staining. Sections (5 μm) were cut in a cryostat and mounted on Star Frost adhesive glass slides (Knittelgläser, Braunschweig, Germany) that were stored at -70°C until use for immunohistochemical analysis.

Antibodies

The following primary mouse monoclonal antibodies (mAbs) were used: biotin-conjugated anti-CD1c (BDCA1; IgG2a, Miltenyi Biotec, Bergisch Gladbach, Germany), biotin-conjugated anti-CD304 (BDCA4; IgG1, Miltenyi Biotec), anti-CD1c (BDCA1; IgG2a, Miltenyi Biotec), anti-CD304 (BDCA4; IgG1, Miltenyi Biotec), FITC-conjugated anti-CD1c (BDCA1; IgG2a, Miltenyi Biotec), FITC-conjugated anti-BDCA2 (IgG1, Miltenyi Biotec), anti-CD11c (IgG1, BD Pharmingen, San Diego, CA), PE-conjugated anti-CD123 (IgG1, BD Biosciences, San Jose, CA), anti-CD83 (IgG1, BD Pharmingen), anti-CD3 (IgG1, BD Biosciences), anti-CD8 (IgG1, BD Biosciences), anti-IL-12p70 (IgG1, R&D Systems, Minneapolis, MN), anti-IL-15 (IgG1, Diaclone SAS, Besançon, France), anti-IL-18 (IgG1, MD Biosciences, St. Paul, MN), and anti-IFN-α and IFN-β (both IgG1, PBL Biomedical Laboratories, Florence, Italy). Polyclonal rabbit anti-human IL-23p19 subunit was kindly provided by Dr. J. Pirhonen (Department of Microbiology, National Public Health Institute, Helsinki, Finland).

Immunohistochemical staining

Acetone-fixed cryosections were incubated with mAbs against CD1c (BDCA1) or CD304 (BDCA4) for 1 h at room temperature after blocking endogenous peroxidase activity with H₂O₂ and sodium azide (NaN₃). As negative controls, the primary antibodies were omitted or irrelevant isotype-matched antibodies were applied. After incubation with goat anti-mouse horseradish peroxidase (HRP)-conjugated (DakoCytomation, Glostrup, Denmark) for 30 min at room temperature, sections were incubated for 15 min with biotinylated tyramine (Perkin Elmer, Boston, MA), followed by an incubation with streptavidin-HRP (strept-HRP, DakoCytomation). Peroxidase activity was revealed using amino-ethylcarbazole (AEC) substrate kit (SK-4200, Vector laboratories, Burlingame, CA). With this procedure CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC stained red. Sections were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MA), dried and mounted with glycerol/gelatine.
For double immunohistochemical stainings with CD1c (BDCA1) or CD304 (BDCA4) in combination with CD3 or CD8, and CD11c or CD123 respectively, the sections were incubated overnight at 4°C with either biotin-conjugated anti-BDCA1 or with biotin-conjugated anti-BDCA4 as primary mAbs. After incubation with strep-HRP for 30 min at room temperature sections were incubated for 15 min with biotinylated tyramine, followed by incubation with strep-HRP for 30 min. Following a 15 min incubation with 10% normal mouse serum, anti-CD3, anti-CD8, anti-CD11c or anti-CD123 mAbs were applied to the sections and incubated for 1 h at room temperature followed by incubation with alkaline phosphatase (AP)-conjugated goat anti-mouse for 30 min. Peroxidase activity was revealed as stated above and AP activity was revealed using alkaline phosphatase substrate III kit (SK-5300 Vector laboratories).

Immunofluorescence staining

For double immunofluorescence staining, sections were first incubated with FITC-conjugated primary mAbs against anti-BDCA1 or BDCA2 followed by incubation with rabbit anti-FITC (DakoCytomation) and with Alexa-488 conjugated goat anti-rabbit (Molecular Probes Europe, Leiden, The Netherlands). After blocking with normal mouse serum, the sections were incubated with the mouse monoclonal antibodies against CD83, IL-12p70, IL-15, IL-18, IFN-α or IFN-β, or with the rabbit polyclonal against IL-23p19. After incubation with Alexa-594-conjugated goat anti-mouse or with Alexa-594-conjugated goat anti-rabbit (Molecular Probes Europe), the slides were analyzed using a fluorescence microscope (Leica DMRA, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands). For coexpression of CD83 and mDC or pDC 12 patients with RA, 5 patients with OA and 7 patients with PsA were analyzed. For the expression of cytokines by mDC and pDC 5 patients with RA were analyzed. To quantify the data, the numbers of double positive staining cells were counted in a minimum of 6 microscopic fields and the percentage of double positive cells was calculated. The selection of these patients was based on the presence of mDC and/or pDC in the synovium.

Quantification of CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC numbers by digital image analysis

All sections were coded and randomly analyzed by computer-assisted image analysis. For all markers, 18 high-power fields were analyzed. The images of the high-power fields were analyzed using the Qwin analysis system (Leica, Cambridge, UK), as described previously (18).

Statistical analysis

Data are expressed as mean ± SEM. Differences between two groups were analyzed for statistical significance with the Mann-Whitney U test using the GraphPad InStat® software (version 3.00; GraphPad InStat, Inc., San Diego, CA). A P value <0.05 was considered as the level of significance.
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* RA = rheumatoid arthritis; PsA = psoriatic arthritis; OA = osteoarthritis; ESR = erythrocyte sedimentation rate; CRP = C reactive protein; RF = rheumatoid factor
RESULTS

Patients
Clinical data of the patients used in this study are presented in Table 1. The mean duration of the disease was 38.8 ± 49.0 (05-180) months in RA patients, 196.2 ± 135.1 (12-456) months in OA patients, and 81.6 ± 97.3 (2-240) months in PsA patients [mean ± SD (range)].

Inflamed synovium contains comparable numbers of mDC and pDC
Although both mDC and pDC have been identified in synovial fluid within the inflamed synovial compartment by several groups (19,20), analysis of synovial tissue has until recently been hindered by complex methodologies and lack of specific surface markers. To gain insight into the in vivo expression of CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC in synovial tissue we performed immunohistochemical analysis. In normal healthy controls, CD1c (BDCA1)+ and CD304 (BDCA4)+ DC are (virtually) absent in synovial tissue (data not shown). Immunohistochemical analysis revealed CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC dispersed throughout the synovial sublining, but not in the intimal lining layer in all forms of synovitis (RA, PsA and inflammatory OA) (Figure 1A). Furthermore, the mean number of mDC and pDC in synovium did not differ significantly between disease groups (Figure 1B). Interestingly,

Figure 1. Expression of CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC in synovial tissue from patients with RA, OA and PsA. See Color figures
(A) Representative sections of synovial tissue are shown (original magnification x 400). (B) No significant differences were observed between the numbers of both mDC and pDC present in RA, PsA and inflammatory OA synovia. (C) In RA, PsA, and inflammatory OA synovia the numbers of pDC are significant higher compared to mDC. In (B) and (C) sections were analyzed using computer assisted digital image analysis. Results are shown as mean numbers of positive cells/mm²±SEM of 22 patients with RA, 10 patients with PsA and 11 patients with inflammatory OA. Differences between two groups were analyzed for statistical significance with the Mann-Whitney U test (*P<0.05, **P<0.01, ***P<0.001).
within each diagnostic group the numbers of pDC were significantly higher compared to mDC (RA, $P<0.001$; PsA, $P=0.002$; Inflammatory OA, $P=0.0003$) (Figure 1C).

Phenotype of mDC and pDC in RA synovial tissue
To confirm the myeloid and plasmacytoid DC phenotype in rheumatoid synovium, we analyzed the coexpression of CD11c (myeloid marker) or CD123 (IL-3Rα) by CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC, respectively. CD11c is present on mDC, but not on pDC and CD123 is present on pDC, but not on mDC (Figure 2, arrows). Moreover, by using specific antibodies to accurately define DC subsets (CD1c and CD304), we found that not all CD11c+ cells belong to the CD1c (BDCA1)+ mDC subset and not all CD123+ cells belong to the CD304 (BDCA4)+ pDC subset.

Inflamed synovium contains immature mDC and pDC
Immature DCs (iDC) are characterized by a high capability for antigen capture and processing, but low T cell stimulatory capability. DC maturation, promoted by inflammatory mediators such as TNF-α and IL-1β, is characterized by up-regulation of costimulatory and maturation molecules and concurrent capacity to uniquely initiate specific immune responses by stimulating T helper cells (23). Therefore, we analyzed the maturation status (using CD83 as a marker of DC maturation) of both mDC and pDC in synovial tissue of patients with RA in comparison to PsA and inflammatory OA patients. No significant differences were observed in the percentage of mDC and pDC expressing CD83 between RA patients compared to PsA patients and inflammatory OA (Figure 3A and Table 2). In general, the mean percentage of mature CD83+ DC (mDC 9.9 ± 3.6 and pDC 21.0 ± 3.6, mean ± SEM) was low in all patient
Myeloid and plasmacytoid DC in RA synovium exhibit distinct cytokine profiles

groups (12 RA + 7 PsA + 5 OA), indicating that the majority of mDC and pDC present in the synovium display an immature phenotype. A representative double immunostaining of RA synovium from one patient is shown in Figure 3B. Of interest, in RA synovium the percentage of pDC expressing CD83 was significantly higher \((P < 0.05)\) compared to mDC expressing this maturation marker (Table 2). The results of the analysis were confirmed using DC-LAMP as an alternative marker of DC maturation (data not shown).

![Figure 3](image)

**Figure 3. Maturation status of mDC and pDC in RA, PsA and inflammatory OA synovia.** See Color figures

(A) The percentage of both mDC and pDC coexpressing the DC maturation marker CD83 in RA synovium did not differ significantly compared to PsA and inflammatory OA synovia. (B) A representative double immunofluorescence staining of RA synovium from one patient is shown. CD1c (BDCA1)\(^+\) and CD303 (BDCA2)\(^+\) DC in green, CD83\(^+\) cells in red and double positive cells in yellow (Original magnification x400).

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mDC and pDC are localized in lymphocyte aggregates in RA synovial tissue

The presence of both immature and mature DC in synovial tissue was of great interest given the former’s intrinsic ability to take up antigen and the latter’s capacity to uniquely initiate specific immune responses by stimulating T helper cells (6). Hence, we analyzed the distribution of mDC and pDC in RA synovial tissue in relation to T cell infiltration. To this end, double
immunohistochemistry was performed using antibodies against CD3 and CD8, but not CD4 as both CD1c+ and CD304 (BDCA4)+ DC also express the CD4 antigen (21). Both mDC and pDC can be identified in close proximity to clusters of CD3 and CD8 positive cells (Figure 4).

Differential expression of cytokines by mDC and pDC in RA synovial tissue

RA is considered a predominantly Th1 skewed disorder. Given the robust ability of both mDC and pDC to release a variety of inflammatory cytokines and subsequently drive the direction of the adaptive T cell response, we decided to analyze the expression of IL-12p70, IL-15, IL-18, IL-23-p19, IFN-α and IFN-β by DC in RA synovial tissue. In Figure 5A, representative sections of RA synovial tissue staining are shown. Quantification of mDC and pDC expressing the aforementioned cytokines is depicted in Figure 5B. As expected, IL-12p70 is expressed by mDC only and not by pDC. Conversely, IFN-α is expressed by pDC only and not by mDC. Although the expression of IFN-β by pDC tends to be higher compared to mDC, the difference did not reach statistical significance (P=0.0813). IL-15 is expressed in relatively equal proportions by both subsets. Interestingly, the percentage of pDC that express IL-18 is significantly higher (P=0.002) compared to mDC. Conversely, IL-23p19 expression by mDC was significantly higher (P=0.0273) compared to pDC.

Figure 4. mDC and pDC are localized in lymphocyte aggregates in RA synovial tissue. See Color figures

Double immunohistochemistry stainings were performed to investigate the distribution of CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC in relation with CD3 and CD8 positive T cells. Both mDC and pDC in RA synovium can be identified in close proximity to clusters of CD3 and CD8 positive cells. A representative double immunostaining of RA synovium from one patient is shown. (Original magnification x400).
Myeloid and plasmacytoid DC in RA synovium exhibit distinct cytokine profiles

Figure 5. Expression of cytokines by mDC and pDC in RA synovium. See Color figures.

Double immunofluorescence stainings were performed to investigate the expression of IL-12p70, IL-15, IL-18, IL-23p19, IFN-α, or IFN-β by CD1c (BDCA1)+ mDC and CD303 (BDCA2)+ pDC. (A) A representative double immunofluorescence staining of RA synovium from one patient is shown. CD1c (BDCA1)+ and CD303 (BDCA2)+ DC in green, cytokines in red and double positive cells in yellow (Original magnification x400). (B) Quantification of cytokine expression by mDC and pDC. Differences between two groups were analyzed for statistical significance with the Mann-Whitney U test (*P<0.05, **P<0.01, ***P<0.001).
DISCUSSION

In this study, we have performed for the first time a quantitative and comparative analysis of the distribution and phenotype of mDC and pDC within, and between, RA, PsA and inflammatory OA. Furthermore, we show that mDC and pDC within RA synovium possess distinct and unique cytokine profiles.

Although enrichment of DC in RA and OA synovial tissue has previously been reported (19,20), the considerable phenotypic, morphologic and functional variations between DC subsets related to maturation and tissue localization has not been analyzed. This partly reflects difficulty in adequately identifying DC subsets in tissues - most methodologies rely on complex combinations of antibody markers. Recently, novel markers useful in human DC studies have been defined that resolve these issues (21). We therefore utilized CD1c and CD304, rather than the less specific CD11c and CD123, to more accurately identify mDC and pDC respectively.

We identified mDC and pDC infiltration to the synovial sublining in RA, PsA and inflammatory OA synovia. The magnitude of this infiltrate did not differ significantly between disease entities suggesting a non-specific inflammatory phenotype. Consistent with this, mDC and pDC were absent from healthy synovium (data not shown) and pDC have not previously been identified in OA synovium (19,20). It is therefore most probable that mDC and pDC are recruited from the circulation to the synovial compartment in response to inflammatory stimuli, supported by previous observations that mDC and pDC are significantly decreased in the peripheral blood of RA patients (22). The possibility that in situ differentiation of either subset driven by the diseased microenvironment, contributed to, or indeed accounted for, the synovial accumulation of both mDC and pDC cannot be discounted. Of particular interest, we show for the first time the remarkable observation that pDC numbers are significantly higher in inflamed synovia compared to mDC. The repercussions of this observation are as yet unclear, but may be indicative of destructive joint disease.

DC have been identified in the peripheral blood, synovial fluid and synovial tissue in RA (15,23) and the DC present in rheumatoid synovium have both an activated phenotype and increased APC activity (24,25). Although the coexistence of both immature and mature DC in RA synovium has been reported earlier (26), there is poor understanding of how DC phenotype differs between inflammatory arthropathies. Here we show that the majority of the mDC and pDC present in RA, PsA and inflammatory OA synovia are immature, as established by low expression of the maturation markers CD83 and DC-LAMP (data not shown). These findings are consistent with our observation that DC maturation is incomplete in the inflamed synovial compartment (synovial fluid) (22). Surprisingly, in RA synovium the number of mature pDC was significantly higher compared to mDC. The large pool of iDC present in RA synovium might be recruited from the blood in order to mature locally and drive synovial inflammation. It is possible that these two DC subsets perpetuate the immune response in RA synovium via
the continuous presentation of arthritogenic peptides to T cells since they are both localized in T cell lymphocyte aggregates. Such events have been linked to the autoimmune process in lupus erythematosus and autoimmune diabetes (15) and could perpetuate autoimmune responses, even in the absence of the original instigating antigen. Therefore, the T cell-DC clusters present in RA synovium might be involved in specific antigen presentation and/or other pathophysiological interactions between T cells and DC that are similar, to some extent, to the physiological cell-to-cell interactions in the secondary lymphoid tissues.

Here we show for the first time that mDC and pDC produce locally (RA synovium) distinct cytokine profiles. During the acute and chronic inflammatory response, cytokines convey pro- and anti-inflammatory signals between and within cells via specific receptor molecules and as such are crucial in the pathogenesis of RA. In this respect, the RA synovial tissue is associated with an increased production of an array of cytokines by several cell types (27,28). As expected, IL-12p70 was confined to mDC. IL-12 production may contribute to local tissue inflammation and induce or enhance IFN-γ production by NK cells and effector CD4+ T cells (29). The role of IL-12 in RA has been suggested by several studies. Expression of IL-12 has been shown in the synovial tissue (30,31) and it is also produced by monocyte-derived DC (32,33) of RA patients. Moreover, neutralization of IL-12 in the murine collagen-induced arthritis (CIA) model dramatically attenuated severity of arthritis (34), commensurate with an important role for mDC-derived IL-12 in RA pathology.

IL-15 manifests numerous biologic activities shared with other cytokines, including the stimulation of proliferation and survival of activated T cells, chemotraction of T cells, and costimulation of proliferation and immunoglobulin synthesis by human B cells (35,36). We and others previously demonstrated IL-15 expression on macrophages, endothelial cells, and fibroblast-like synoviocytes in RA synovial tissue, where it can exert broad functional effects in chronic synovitis (37-39). Moreover, targeting IL-15 has proven therapeutically beneficial in RA (40). We show that in addition to macrophages, endothelial cells, and fibroblast-like synoviocytes, mDC and pDC release IL-15 locally. This production is likely integral to the perpetuation of synovial inflammation as it has been demonstrated that DC produce IL-2, required to enable T cell contact and proliferation, which is tightly co-regulated with the expression of IL-15 (41). In addition, IL-15 is sufficient to drive monocyte conversion to mDC (42) and may therefore represent a local cytokine-mediated feedback loop whereby DC IL-15 release promotes local mDC maturation.

We show that in RA synovium, pDC are the main producers of IL-18 compared to mDC. IL-18 and its inducer IL-12 have multiple biological activities that are important in generating Th1 responses and inflammatory tissue damage. IL-18 produced by macrophages (43) and DC in RA synovium induce T cells to produce more IFN-γ by enhancing the IL-12 effect. IL-18 (31) and IL-12 present in RA synovium act synergistically and induce Th1 responses (44) observed in RA. Furthermore, RA synovial expression of IL-18 is accompanied by the coexpression of IL-1β and TNF-α and was associated with local inflammation (45). Interestingly, IL-18 has been
shown to recruit mDC (46) and pDC (47) to areas of inflammation, in particular under Th1 cytokine conditions where IFN-γ is increased such as in RA. This mechanism may also explain the preferential accumulation of pDC in RA synovium.

We observed that in RA synovium mDC are the main producers of IL-23 compared to pDC. IL-23 promotes the expansion of IL-17-producing cells (Th17) (48), that are involved in RA pathology. In this respect, elevated levels of IL-17 have been detected in the synovial fluid from patients with RA, but not with OA (49,50) and explants of the rheumatoid synovium were found to express and release IL-17 (51). Moreover, IL-23 also play a crucial role in CIA mice, as p19-deficient mice do not develop any clinical signs of joint or bone pathology (52). Thus, in view of the above-mentioned observations, mDC and, to a lesser extent pDC, derived IL-23 may contribute to the expansion of Th17 cells in RA synovium and contribute to RA pathology.

Consistent with their main function (6), here we show that RA synovial pDC are the main producers of IFN-α/β. Type I IFNs enhance humoral autoimmunity, promote isotype switching (53,54), and potently activate autoreactive T cells (55). Type I IFNs are pleiotropic cytokines with dual effects. They contribute to the development of human Th1 cells (56) and paradoxically, they directly inhibit the production of IL-12 by mature DC and reduce Th1 development (57). Moreover, IFN-β has been shown to reduce inflammation and lessen the frequency of multiple sclerosis attacks (58). We propose that type I IFNs produced by RA synovial pDC located in the vicinity of B cells (data not shown) and T cells might induce the development of (auto)antibody-producing plasma cells and activation of autoreactive T cells, respectively. Furthermore, in RA synovium the reduced frequency of mDC compared to pDC might be explained by the enhanced expression of type I IFNs (59) (IFN-β reduces the expression of myeloid markers) (60).

Our results indicate that synovial DC may play an important role in synovial inflammation possibly via stimulation of memory T cells. Moreover, these findings are in line with the concept that in RA patients are characterized by a balance towards Th1 responses (61) due to the release of proinflammatory and Th1-inducing cytokines by synovial DC. In this respect, IL-12 together with IL-18 may induce/enhance IFN-γ production by effector T cells, type I IFNs may play a role in (auto)antibody production by B cells and IL-23 the induction of the newly described Th17 cell subset. Conceivably, immunomodulation by targeting synovial DC might provide a novel antirheumatic strategy.
REFERENCES


Chapter 8

Uncoupling of DC maturation and CCR7 expression

Sander W. Tas¹, Esther C. de Jong², Najat Hajji¹ and Paul P. Tak¹

¹Div. of Clinical Immunology and Rheumatology and ²Dept. of Cell Biology and Histology, AMC/University of Amsterdam, The Netherlands.
ABSTRACT

Dendritic cells (DC) play a crucial role in the induction of immune responses. DC maturation and cytokine production are NF-κB dependent, therefore we hypothesized that blocking NF-κB activity in DC by caffeic acid phenethyl ester (CAPE) could inhibit the maturation of DC and thereby modulate T cell immune responses. Monocyte-derived DC were incubated with CAPE in the presence of the maturation factors LPS or CD40L. DC were analyzed for NF-κB activity, surface marker expression, cytokine production and interaction with naive T cells. Furthermore, the migratory capacity of these cells was studied in a chemotaxis assay. NF-κB blockade by CAPE resulted in strongly reduced maturation of DC. In addition, IL-12p70 production was blocked, whereas IL-10 production was increased. Interestingly, CAPE treatment resulted in increased CCR7 expression both in monocyte-derived DC and naturally occurring DC, which correlated with an enhanced migration towards the CCR7 ligands CCL19 and CCL21. Coculture with naive T cells resulted in reduced proliferation and differentiation of these cells. NF-κB inhibition in DC using CAPE results in an immature phenotype with increased CCR7 expression, reduced inflammatory cytokine production and decreased proliferation and differentiation of naive T cells. The enhanced CCR7 expression and migratory capacity may promote migration to draining lymph nodes and presentation of antigen in the absence of co-stimulation in vivo, leading to tolerance. This hypothesis is supported by the finding that interaction of CAPE-treated DC with T cells results in less proliferation and differentiation into Th1 (effector) cells. These data strongly suggest that targeting NF-κB in DC could be beneficial for immunotherapy in the treatment of autoimmune diseases like arthritis or after transplantation.
INTRODUCTION

Dendritic cells (DC) are key regulators of the immune response that are able to promote or suppress T cell activation (1,2). They are potent antigen presenting cells (APC) DC with the unique ability to stimulate primary immune responses via the instruction of naive T cells in addition to boosting secondary immune responses. DC are not only capable of inducing T cell immunity, but can also induce T cell tolerance (3). Various DC features like the expression of various surface molecules and cytokine production, but also environmental factors, determine the fate of naive T helper cells that interact with these cells (reviewed in (4)).

DC maturation can be induced by many different stimuli and is characterized by increased expression of co-stimulatory molecules and MHC class II, together with pro-inflammatory cytokines. Concomitantly, these DC frequently express the chemokine receptor (CCR)7 that enables DC to migrate to lymph nodes and interact with T cells. Little is known about regulation of CCR7 expression, as the CCR7 promoter has not been characterized (reviewed in (5)). Although CCR7 expression often correlates with the DC maturation status, CCR7 expression can also be induced independently of maturation (6-10). The transcription factor NF-κB plays a key role in the regulation of inflammatory responses (11), including DC development, maturation and APC function (12). NF-κB activation not only controls the expression of MHC class II and co-stimulatory molecules, but also the production of T cell instructing cytokines like IL-12 (13). Consequently, NF-κB inhibition results in a reduced capacity of DC to stimulate T cells (reviewed in (14)). This information could potentially be applied to utilize DC for immunotherapy of unwanted adaptive immune responses seen after transplantation or in auto-immune diseases like rheumatoid arthritis (RA), multiple sclerosis and inflammatory bowel disease (15).

NF-κB is a family of transcription factors containing five members, including RelA (p65), NF-κB1 (p50; p105), NF-κB2 (p52; p100), c-Rel and RelB, that function as homo- or heterodimers. In most resting cells, NF-κB dimers are retained in the cytosol complexed with inhibitory IκB proteins. Upon activation, IκB is phosphorylated by IκB kinase (IKK), leading to degradation, thereby releasing NF-κB followed by nuclear translocation and binding to κB enhancer elements of target genes (reviewed in (16)). NF-κB activation in response to pro-inflammatory signals is dependent on this classical pathway and the main activated form of NF-κB induced by this pathway is a heterodimer of p50 and the transactivating subunit p65 (17).

Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant derived from propolis from honey bee hives that has been demonstrated to have immunomodulatory properties (18-21). CAPE exerts its anti-inflammatory effects in part by anti-oxidant activities (22) and via inhibition of lipoxygenase (23) and nitric oxide synthase enzyme activity (24). Consequently, it is possible
that CAPE exerts its effects by inhibiting the production of reactive oxygen species (25,26). However, it has recently been demonstrated that CAPE also acts as a potent inhibitor of NF-κB activation by preventing IkBα degradation (27) and blocking of p65 nuclear translocation and DNA binding (28). Alternatively, the inhibition of NF-κB activation by CAPE may also be attributed to the ability of caffeic acid esters to alter the cellular redox state (29,30).

As CAPE has been described as a strong inhibitor of the NF-κB pathway we investigated its effects on DC biology and subsequent T cell activation. We hypothesized that CAPE-mediated NF-κB inhibition in DC results in an immature phenotype with reduced T cell stimulatory capacity that may induce tolerance. Throughout our study we used human monocyte-derived DC, as the level of constitutive NF-κB activity in these cells is low and can be rapidly induced and up-regulated following stimulation (31).

In this study we show that CAPE arrests DC in an immature state despite stimulation with LPS or CD40L. Surprisingly, CAPE treatment increased functional CCR7 expression, resulting in enhanced migration of DC to CCR7 ligands. Furthermore, treatment of DC with CAPE resulted in reduced T cell proliferative capacity in the MLR and reduced Th1/Th2 polarization of naive T cells.

MATERIAL & METHODS

Antibodies, cytokines, and reagents

Human IL-4 (20 x 10^8 U/mg) was obtained from Strathmann Biotec AG (Hannover, Germany). Human rGM-CSF (sp. act. 1.11 x 10^7 U/mg) was a gift of Schering-Plough (Uden, The Netherlands). Mouse mAbs to phosphorylated (ph) and total IkBα were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal rabbit anti-p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Clobetasol-17-propionate (CP; Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in ethanol and used at a final concentration of 10^{-7} M. NEMO binding domain (NBD) peptides were synthesized as described previously (32) and subsequently dissolved in DMSO to stocks of 50 mM. NBD or mutant control (MUT) peptides were used at a concentration of 50 μM.

In vitro generation and maturation of DC from monocytes or direct isolation of BDCA1^+ and BDCA4^+ cells from peripheral blood

Monocyte-derived DC were obtained as described previously (33). On day 6, maturation of immature DC was induced by the addition of LPS (100 ng/ml; Sigma, Zwijndrecht, The Netherlands) or CD40 ligand (CD40L)-expressing mouse plasmacytoma cells (irradiated J558 cells, 1:1 ratio with DC; a kind gift from Dr. P. Lane, University of Birmingham, Birmingham, U.K.).
After 48 h, full maturation into CD1a^+CD83^+ mature effector DC was confirmed by flow cytometric analysis. BDCA1^+ and BDCA4^+ cells were isolated from peripheral blood using specific isolation kits from Miltenyi Biotec (Bergisch Gladbach, Germany). BDCA1^+ and BDCA4^+ cells were cultured in the presence of GM-CSF and IL-3 respectively. DC were incubated for 2 h in the presence or absence of CAPE (25 μg/ml) prior to induction of maturation or stimulation by LPS or CD40L. All subsequent tests were performed after harvesting and extensive washing of the cells to remove all factors. In some experiments CCL19 (ELC, MIP3β) or CCL21 (SLC, 6Ckine); 50 ng/ml (R&D Systems, Abbingdon, Oxon, UK) was added to the culture during the last 16 h of maturation.

**Western blotting**

After the indicated times of incubation and stimulation cells were washed twice with ice-cold PBS to remove all serum proteins and then lysed in 1x SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE on a 10% gel, using Rainbow-colored protein molecular weight markers (Amersham, Little Chalfont, U.K.) as a reference, and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in TBS containing 2% non-fat dry milk (Bio-Rad, Hercules, CA), Na_3VO_4 (2mM) and 0.05% Tween 20 during 1 h. Detection of phosphorylated and unphosphorylated IκBα was performed by incubating the membranes with a primary antibody against the protein of interest overnight at 4°C. The membranes were subsequently washed and incubated with the appropriate secondary Ab in TBS containing 2% non-fat dry milk, Na_3VO_4 (2mM) and 0.05% Tween 20 for 1 h at room temperature, and after extensive washing assayed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfront, UK). Densitometry was performed using GeneTools software (SynGene, Cambridge, UK).

**Immunofluorescence staining of p65 and HLA-DR and confocal microscopy analysis**

Immature DC were seeded onto LAB-TEK glass chamber slides (Nalgene Nunc International, Naperville, IL) at a density of 500 cells per well and stimulated for 4 h with LPS in the presence or absence of CAPE (25 μg/ml). Subsequently, cells were washed, air-dried and fixed in cold acetone for 10 min. The slides were then washed extensively with PBS and incubated with optimal dilutions of the primary Ab in PBS containing 1% FCS and 1% human serum for 60 min at room temperature, followed by 60 min incubation at room temperature with Alexa 488-conjugated goat-anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) and Cy3-conjugated streptavidin (Sigma) in PBS containing 1% FCS and 1% human serum to detect p65 and HLA-DR, respectively. For each fluorochrome label, negative control antibodies were included. Finally, the slides were incubated with Hoechst for 5 min to label the nuclei and mounted in Vectashield (Vector Labs, Burlingame, CA) for confocal microscopy analysis. RelA/p65 and HLA-DR were visualized using a Leica TCS SP (Leica Microsystems, Heidelberg,
Germany) confocal system, equipped with an Ar/Kr/HeNe laser combination. Images were taken using a ×40 1.25 NA objective.

Analysis of cell surface molecule expression by flow cytometry
At day 8, the maturated “effector” DC were analyzed for the expression of cell surface molecules by flow cytometry. Mouse anti-human mAbs against the following molecules were used: CD1a (OKT6; Ortho Diagnostic System, Beerse, Belgium), CD83 (HB15a, IgG2b; Immunotech, Marseille, France), CD86 (IT2.2, Pharmingen, Erembodegem, Belgium), and HLA-DR (L234, IgG2a; BD Biosciences, San Jose, CA). Primary mAb were detected by FITC-conjugated goat anti-mouse mAb (Jackson ImmunoResearch Laboratories, West Grove, PA). CCR7 expression was detected using a PE-conjugated anti-CCR7 antibody (Pharmingen, San Diego, CA, USA).

Cytokine production by DC
DC (2 x 10^4 cells/well) were stimulated with CD40L-expressing J558 cells (2 x 10^4 cells/well), in 96-well flat-bottom culture plates (Corning Life Sciences, Schiphol-Rijk, The Netherlands) in IMDM containing 10% FCS in a final volume of 200 µl. Supernatants were harvested after 24 h and stored at -20°C until the levels of IL-12p70, IL-6 and IL-10 were measured by specific solid-phase sandwich ELISA as described previously (34). The detection limit of the assay was 3 pg/ml.

Actin-polymerization assay
The actin-polymerization assay was adapted from previously described methods (35). DC (2.5 x 10^5 cells in a volume of 100 µl medium) were transferred to tubes and the stimulating chemokine, CCL19 or CCL21 (100 ng/ml; R&D Systems, Abbingdon, Oxon, UK) was added for 1 min. Cells were immediately fixed with 50 µl buffered formaldehyde acetone solution and subsequently permeabilized by washing with PBS/0.5% BSA (PBA) containing 0.5% saponine. Cells were then incubated for 30 min with phalloidin–FITC (Sigma Chemical, St Louis, MO, USA) to visualize the F-actin. Cells were washed again in PBA containing 0.5% saponine and analyzed by FACS.

Migration assay
Twenty-nine microliters of chemokine/chemoattractant in medium or medium alone (negative control), were placed in the bottom of the well of a microchemotaxis plate (Neuroprobe, Gaithersburg, MD). The chemokines CCL19 and CCL21 were used in a concentration of 50 ng/ml. DC were loaded with a fluorescent dye, 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxy-fluorescein, acetoxyethyl ester (BCEF-AM) (Molecular Probes, Eugene, OR), and as a positive control, 25 µl of labeled cells were placed in a bottom well. A framed filter (5 µm) was then placed over the microplate and 25 µL of the prepared cells were placed on the top of the
filter. All conditions were tested in triplicates. The plate was then incubated at 37°C in humidified air with 5% CO₂ for 30 min. After incubation, the non-migrated cells were removed from the top of the filter. The chemotaxis chamber was then placed in a multi-well fluorescent plate reader (Perkin Elmer, Boston, MA), and the cells that migrated into the bottom chamber were measured by using the BCEF-AM fluorescence signal. The fluorescent plate reader was configured so that the probe was in a bottom-read position, which allowed for the detection of fluorescence in each well of the chemotaxis chamber.

**Real-time PCR quantification of CCL19 and CCL21**

RNA was first purified using RNeasy kit (Qiagen, Valencia, CA) and oligo-dT17-primed first-strand cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction (RT-PCR) was performed in single wells of a 96-well plate (BioRad, Hercules, CA) in a 25 μl reaction volume. The reaction mixture contained, 12.5 μl SYBR Green mix (Sigma), 1 μl of forward primer, 1 μl of reverse primer, 5.5 μl of ddH₂O, and 5 μl of RNA sample. The following primers were used: 5’-TGGCACCAATGATGCTGAAG-3’ (F) and 5’-CCCTCAGTGTGGTGAACACTAC-3’ (R) for CCL19, and 5’-CTCCATCCCAGCTATCCTGTTC-3’ (F) and 5’-TGCAACCAGACTCCTTGTG-3’ (R) for CCL21. Primers were used at a concentration of 500 nM. All experiments were performed using the real-time iCycler™ PCR platform (Bio-Rad). Thermal cycling conditions were as follows: an initial incubation at 95 °C for 10 min to activate the polymerase followed by 50 cycles of 95 °C for 30 s, 59 °C for 1 min and 72 °C for 45 s, and a final incubation at 72 °C for 10 min. Fluorescence was measured following each cycle and displayed graphically (iCycler iQ Real-time Detection System Software, version 2.3, BioRad, Hercules, CA). The software determined a cycle threshold (Ct) value, which identified the first cycle at which the fluorescence was detected above the baseline for that sample or standard. The mean values from duplicates were used for calculations. Data are expressed as normalized expression obtained by dividing the relative cDNA level of CCL19 or CCL21 for each sample by the relative cDNA level of β-2-microglobulin for the same sample using geNorm software version 3.4 (36).

**Mixed lymphocyte reaction**

Effector DC were also tested for their ability to stimulate allogeneic naive Th cells in a MLR. Highly purified CD4⁺CD45RA⁺CD45RO⁻ naive Th cells (>98% as assessed by flow cytometry) were isolated from PBMC using a CD4⁺ T cell isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), followed by depletion of CD4⁺CD45RO⁺ T cells using PE-labeled anti-CD45RO (Dako, Glostrup, Denmark) and anti-PE-beads (Miltenyi Biotec). Naive Th cells (2.5 x 10⁴ cells per 200 μl) were cocultured in 96-well flat-bottomed culture plates with different concentrations of mature DC. After 5 d, cell proliferation was assessed by the incorporation of [³H]thymidine (Radiochemical Center, Amersham, Little Chalfont, U.K.) after a pulse with 13 kBq per well during the last 16 h, as measured by liquid scintillation counting.
Determination of naive Th cell polarization by DC.

Highly purified CD4+CD45RA+CD45RO- naive Th cells (2 x 10^4 cells/200 µl of IMDM with 10% FCS) were cocultured with 5 x 10^3 effector DC coated with *Staphylococcus enterotoxin* B (Sigma-Aldrich; final concentration, 10 pg/ml) in 96-well flat-bottom culture plates (Costar). At day 5, human rIL-2 (10 U/ml, Cetus, Emeryville, CA) was added, and the cultures were expanded for the next 7 days. On day 12, the quiescent Th cells were counted and equal cell numbers were restimulated with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) for 6 h. During the last 5 h Brefeldin A (10 µg/ml; Sigma-Aldrich) was present to inhibit secretion of intracellularly produced proteins. Single cell IL-4 and IFN-γ production was determined by intracellular flow cytometric analysis. Cells were fixed in 2% formalin (Merck, Darmstadt, Germany), permeabilized with 0.5% saponin (Sigma) and stained with anti-human IFN-γ-FITC and anti-human IL-4-PE (both from BD Biosciences, Franklin Lanes, NJ). The cells were evaluated by FACScalibur (BD Biosciences).

Statistical analysis

Data were analyzed for statistical significance (GraphPad, InStat, version 2.02) using ANOVA or Student’s t test. A p value <0.05 was taken as the level of significance.

RESULTS

CAPE effectively blocks LPS- and CD40L-induced DC maturation

DC maturation is transcriptionally regulated by NF-κB (12) and can be evaluated by examining surface CD83 expression on these cells. We investigated whether pre-treatment with CAPE affected LPS- or CD40L-induced DC maturation. Glucocorticoids (GC) are widely used immunosuppressive drugs that have been demonstrated to exert immunoregulatory effects on DC (37,38). Therefore, we compared the effects of CAPE on DC maturation with the widely used GC clobetasol-17-propionate (CP) (33,39,40) and the highly selective IKKβ inhibitor NEMO binding domain (NBD) peptide (41) that are both known to block DC maturation via inhibition of NF-κB. Immature DC were pre-treated with the indicated NF-κB inhibitors for 2 h followed by LPS or CD40L stimulation for 2 days. Cells were then stained and analyzed by flow cytometry for the expression of surface CD83 as a marker of DC maturation. We found that optimal concentrations of all inhibitors abrogated the LPS-induced up-regulation of CD83, whereas only CAPE and the NBD peptide potently inhibited the up-regulation of CD83 in response to CD40L stimulation (Figure 1). In conclusion, unlike GC, CAPE inhibited both LPS- and CD40L-induced DC maturation. Consequently, we tested whether CAPE exhibited differential effects on other DC functions compared with the selective canonical NF-κB blocking NBD peptide.
CAPE blocks IκBα phosphorylation and nuclear translocation of NF-κB p65 in DC

Since CAPE has been demonstrated to inhibit IκBα phosphorylation in endothelial cells (27), we investigated the potential to do so in DC. Therefore, the phosphorylation status of IκBα in cytoplasmic extracts from DC was determined by Western blot using Abs specific for the phosphorylated form of this protein. Induction of IκBα phosphorylation was readily detected in cytoplasmic extracts prepared from DC stimulated with LPS compared to unstimulated DC (Figure 2A). Pretreatment with CAPE for 2 h significantly inhibited phosphorylation of IκBα following LPS stimulation and reduced the level of phospho-IκBα to the levels observed in unstimulated cells. Comparable results were obtained when DC were stimulated with IL-1β (10 ng/ml), TNFα (10 ng/ml) or CD40L for 30 min (data not shown). Similar to the NBD peptide (41), these results demonstrate that CAPE inhibits IκBα phosphorylation in DC. Since CAPE has been described to inhibit nuclear translocation and DNA binding, and because a block in IκBα phosphorylation should result in sequestration of NF-κB in the cytoplasm and reduced nuclear translocation, we studied the effect of CAPE-treatment on nuclear translocation of the main transactivating NF-κB subunit, p65, in DC. Confocal microscopic analysis of DC that were stimulated with LPS for 4 h showed unambiguous translocation of p65 to the nucleus (Figure 2B). CAPE-pretreatment of these DC resulted in a complete block in nuclear transloca-
tion of p65 in all cells. In addition, HLA-DR expression that has been known to be controlled by NF-κB responsive genes, was also reduced in CAPE treated DC (Figure 2B). These results provide evidence that CAPE-pretreatment of DC blocks nuclear translocation of NF-κB in response to LPS.

Pro-inflammatory cytokine production by DC is inhibited by CAPE

Next, we investigated whether CAPE-mediated NF-κB inhibition has consequences for the production of cytokines. Therefore, CAPE-pretreated DC were stimulated with LPS for 2 days.
Subsequently, DC were stimulated with CD40L for 24 h to determine IL-12p70 production in the supernatant. CAPE-treated DC secreted reduced levels of the T cell stimulatory cytokine IL-12p70 after re-stimulation ($p<0.05$)(Figure 3A). In addition, IL-6 production was significantly decreased in CAPE-treated DC ($p<0.05$). These results are comparable to incubation
of the DC with the NBD peptide (41). Surprisingly, IL-10 production was strongly enhanced after CAPE treatment ($p<0.05$). Similar results were obtained with DC incubated with CAPE and simulated with CD40L. No significant differences in DC viability were observed between treatment and control groups based on flow cytometry (data not shown). These data indicate that CAPE induces a tolerogenic DC phenotype, characterized by reduced pro-inflammatory cytokine production and increased production of the anti-inflammatory cytokine IL-10.

CAPE blocks upregulation of MHC class II and co-stimulatory molecules

The observed reduction in cytokine production after CAPE treatment suggests that NF-$\kappa$B inhibition in DC results in an altered phenotype. In addition to pro-inflammatory cytokine
production, the expression of co-stimulatory molecules and antigen presenting molecules like MHC class II is also transcriptionally regulated by NF-κB (12). Hence, we examined whether pre-treatment with CAPE affected the up-regulation of these molecules after DC activation. Immature DC were pre-treated with CAPE for 2 h followed by LPS or CD40L stimulation for 2 days. Cells were then stained and analyzed by flow cytometry for the presence of surface HLA-DR, CD83, and CD86. We found that CAPE abrogated the up-regulation of HLA-DR \( (p < 0.05) \), CD83 \( (p < 0.05) \), and CD86 \( (p < 0.05) \) both after LPS stimulation (Figure 3B; upper panels) and CD40 ligation (Figure 3B; lower panels). The reduced surface expression of HLA-DR on CAPE treated DC as quantified by FACS analysis confirmed the earlier findings by confocal microscopy (see Figure 2B). These data show that NF-κB inhibition by CAPE alters the maturation state, MHC class II and co-stimulatory molecule expression of stimulated DC, which is likely to have major consequences for the capacity of these DC to present antigens to naive T cells.

**CAPE results in increased functional CCR7 expression in DC**

DC maturation requires NF-κB activation and is usually (but not always) accompanied by CCR7 up-regulation, however, the mechanisms involved are largely unknown. A recent report indicated that CCR7 expression in DC is regulated via a TREM-2/DAP12-mediated pathway that is extracellular signal-regulated kinase (ERK) dependent, but independent of NF-κB (7). Therefore, we investigated the effects of NF-κB inhibition on DC CCR7 expression. Surprisingly, CAPE resulted in an increase in the expression of CCR7, both in LPS- and CD40L-stimulated monocyte-derived DC (Figure 4A). To substantiate these findings, we investigated whether the observed increase in CCR7 expression following CAPE treatment could be found in natural occurring DC as well and compared this to the effects of the NBD peptide on CCR7 expression. Therefore, myeloid DC (BDCA1\(^+\)) and plasmacytoid DC (BDCA4\(^+\)) were isolated...
from peripheral blood, treated with CAPE or NBD/MUT peptide and stimulated with CD40L. We also observed a strong increase in CCR7 expression in these cells (Figure 4B), underlining that CAPE results in elevated expression of CCR7 in naturally occurring DC as well. The increased expression of CCR7 on CAPE-treated DC could change the migration pattern of these cells and enable them to enter secondary lymphoid organs or inflamed tissues. In order to migrate, cells must change their cytoskeleton by polymerization of the F-actin component (42). Phalloidin specifically associates with polymerized actin, the levels of which transiently increase following chemokine stimulation (43). To test whether the increased CCR7 expression on CAPE-treated DC is functional, we quantified the increase in F-actin after stimulation with the CCR7 ligands, CCL19 and CCL21, using fluorescent phalloidin. As shown in Figure 4C, actin polymerization was induced within 1 min after stimulation with CCL21 or CCL19. These data show that the chemokine receptor CCR7, expressed on the surface of CAPE treated DC, was sensitive for chemokine stimulation, resulting in changes in the cytoskeleton.

Figure 6. CAPE treatment of DC results in reduced expression of CCL19 and CCL21, however the increased CCR7 expression does not result from low levels of these ligands.

(A) CAPE blocks basal and CD40L-induced up-regulation of the CCR7 ligands CCL19 and CCL21 in monocyte-derived DC. DC were cultured with LPS or CD40L in the presence or absence of CAPE. After 2 days, DC were analyzed for the expression of the chemokines CCL19 and CCL21 by real-time PCR. Data are normalized to β-2-microglobulin expression and representative of three independent experiments. (B) Addition of CCR7 ligands to CAPE-treated DC does not alter CCR7 expression. DC were cultured with LPS or CD40L in the presence of CAPE and CCL19 and/or CCL21 was added to the culture during the last 16 h of maturation. DC were subsequently analyzed for the expression of cell surface CCR7 by flow cytometry. Data represent relative CCR7 levels and are expressed as mean ± SEM from three different experiments performed in duplicate.
Uncoupling of DC maturation and CCR7 expression

CAPE treated DC specifically migrate towards a chemokine gradient

To test the migratory properties of CCR7+ CAPE-treated DC towards CCR7 ligands, an in vitro transwell system was used. Initial experiments showed that the optimal concentration of CCL19 and CCL21 was 50 ng/ml (data not shown). The migration assays were performed using DC cultured for 2 days with LPS or CD40L. The percentage of specific migration, corrected for the spontaneous migration, is shown. The specific migration of CAPE-treated DC was higher than that of untreated DC, reflecting the increased CCR7 expression, both in LPS-stimulated (Figure 5A) and in CD40L-stimulated DC (Figure 5B). These data show that CCR7+ CAPE-treated DC migrate better towards the chemokines CCL19 and CCL21, indicating that the increased CCR7 expression has functional consequences for DC biology.

CAPE-induced CCR7 expression does not result from altered CCL19 or CCL21 production

To investigate the underlying mechanism of the increased CCR7 expression after CAPE treatment, we determined expression of the CCR7 ligands CCL19 and CCL21 in CAPE-treated DC. Real-time PCR analysis revealed that CD40L stimulation of DC induced both CCL19 and CCL21 (Figure 6A). CAPE resulted in decreased expression of both CCL19 and CCL21, in unstimulated and in LPS- or CD40L-stimulated DC. To test whether reduced expression of the ligands may be responsible for the increased CCR7 expression, we added exogenous CCL19, CCL21 or the combination to the culture and evaluated CCR7 expression on CAPE-treated DC. Addition of CCR7 ligands to the culture did not result in alterations in CCR7 expression (Figure 6B), indicating that the underlying mechanism of the increased CCR7 expression after CAPE-treatment is not a rebound phenomenon due to reduced expression of the ligands CCL19 and CCL21.
Chapter 8

CAPE inhibits the capacity of DC to induce T cell proliferation

To examine the potential of CAPE-treated DC to prime naive T cells, immature DC were pre-treated with CAPE, stimulated with LPS for 2 days and mixed with allogeneic naive CD4+ T cells. T cell proliferation was measured by [3H]thymidine incorporation. We found that CAPE treatment of DC results in dramatically reduced capacity to induce T cell proliferation compared to control DC at all DC:T cell ratios ($p<0.05$)(Figure 7A). A similar reduced capacity to induce T cell proliferation was observed in CD40L stimulated DC ($p<0.05$)(Figure 7B). Direct effects of CAPE on T cell proliferation were excluded by extensive washing in the experiments. These data are in agreement with the observed reduced IL-12p70 production and the inhibited up-regulation of surface molecules such as HLA-DR, CD80 and CD86 that are essential for effective induction of T cell proliferation.

Reduced Th polarization of naive T cells by CAPE treated DC

Because T cell proliferation was significantly reduced in the MLR, we further investigated which type of immune response is induced by CAPE treated DC. Hence, we loaded CAPE-treated LPS- or CD40L-stimulated DC with the superantigen *Staphylococcus aureus* enterotoxin B (SEB) and cocultured them with naive Th cells. After 12 days we re-stimulated the T cells and single cell IL-4 and IFN-γ production was measured by intracellular flow cytometric analysis to determine the percentage of IL-4 and IFN-γ producing T cells after DC instruction. CAPE mediated NF-κB blockade in LPS-stimulated SEB-primed DC resulted in a markedly reduced development of both IFN-γ and IL-4 producing Th cells from naive precursors, whereas no
difference in the development of IL-4 or IFN-γ producing Th cells was observed when control DC were used to stimulate naive Th cells (Figure 8A). CD40L-stimulated DC are known to favor Th1 polarization. CAPE resulted in a near complete block in Th1 polarization, but also inhibited development of IL-4 producing Th cells (Figure 8B). These data show that, in addition to reduced T cell proliferation, CAPE treatment of LPS or CD40L stimulated DC blocks Th cell polarization by inhibiting both Th1 and Th2 differentiation, resulting in a non-polarized Th phenotype.

**DISCUSSION**

The current study reveals that CAPE-treated DC exhibit a number of unique properties to mediate immunoregulation. We found that CAPE treatment of DC blocked NF-κB activation, resulting in a tolerogenic phenotype with reduced surface expression of MHC class II and costimulatory molecules, and decreased production of the T cell instructing cytokine IL-12, but increased IL-10 production. Surprisingly, CAPE treatment concomitantly increased functional CCR7 expression, resulting in enhanced migration of these cells to the CCR7 ligands CCL19 and CCL21. In addition, interaction of CAPE-treated DC with naive T cells resulted in significantly reduced T cell responses.

Blockade of NF-κB activation generally alters the antigen presenting capacity of DC resulting in the induction of T cell tolerance (reviewed in (14)). We compared the effects of CAPE to optimal concentrations of CP or the NBD peptide, which have been described to block NF-κB activation (41). GC and CAPE blocked LPS-induced maturation equally well, but only CAPE strongly inhibited CD40L-induced DC maturation, just like the NBD peptide. Subsequently, we demonstrated that CAPE blocked both IκBα phosphorylation and nuclear translocation of NF-κB p65 in DC. This resulted in reduced production of the pro-inflammatory cytokines IL-6 and IL-12. Surprisingly, CAPE treatment resulted in an increase in the production of the immunoregulatory cytokine IL-10. IL-10-producing DC have been demonstrated to induce tolerance before (44-46), yet, the precise mechanisms involved in IL-10 production remain largely unknown. Tolerogenic IL-10-producing DC can be generated by treating the cells with immunosuppressive drugs, such as dexamethasone (47,48), vitamin D(3) analogs (46) or certain other pharmacological compounds that suppress of NF-κB signaling (48,49). NF-κB inhibition in DC does not always result in enhanced IL-10 production (41,50,51), but these cells still induced T cell tolerance. Therefore, the effects of CAPE on IL-10 production may be either NF-κB inhibition-dependent or -independent.

Efficient adaptive immunity depends on the interaction between APC and T cells. Trafficking of these cells is mainly directed by the chemokine system and the expression of adhe-
sion molecules on these immune cells, as well as on stromal cells (52). The combination of both constitutive and inducible production of chemokines and changes in the expression of different chemokine receptors and adhesion molecules bring immune cells together. This intricate network leads to controlled immune cell migration and the initiation or perpetuation of adaptive immunity. The chemokine receptor CCR7 plays an important role in trafficking of both T cells and DC (52,53). Interestingly, CAPE treatment of DC resulted in increased functional CCR7 expression on these cells. CAPE-induced CCR7 was sensitive for CCL19 and CCL21, since stimulation with these CCR7 ligands resulted in changes in the cytoskeleton characterized by increased actin polymerization. In addition, CAPE-treated CCR7 expressing DC migrated better towards CCL19 and CCL21. The increased CCR7 expression was not only observed in monocyte-derived DC, but also in naturally occurring myeloid or plasmacytoid DC, indicating CAPE-induced CCR7 expression is a more general mechanism that could have major implications for the use of this drug \textit{in vivo}.

CCR7 expression often correlates with the DC maturation status, but CCR7 expression can also be induced independently of maturation (6-10). Similarly, CAPE resulted in a block in LPS- and CD40L-induced maturation accompanied by increased CCR7 expression. Although CAPE treatment of DC resulted in robust up-regulation of CCR7, this was not likely to be mediated by NF-κB inhibition as increased CCR7 expression was not, or to a much lesser extent, observed after NF-κB inhibition using corticosteroids (54), vitamin D(3) analogs (54,55), or the highly selective IKKβ inhibitor NBD peptide. In addition, IL-6 has been demonstrated to block both maturation, NF-κB binding activity, and CCR7 expression in DC, therefore the increased CCR7 expression after CAPE treatment cannot be explained by NF-κB inhibition alone (56). The intricate signal transduction networks that control CCR7 expression in DC are extensively studied and suggest that CCR7 may be crucially dependent on MAPK activation, whereas NF-κB is required for DC maturation, but not involved in the up-regulation of CCR7 (reviewed in (57)). Regulated negative cross-talk exists between distinct signaling pathways, such as MAPK and NF-κB (58-61). CAPE may shift the balance in such a way that NF-κB is inhibited, favoring MAPK activation (62). However, this requires further investigation.

The specific CCR7 ligands CCL19 and CCL21 are constitutively produced by cells of the high endothelial venules and stromal cells within T cell areas of lymphoid organs (53), but also in endothelial cells of inflamed tissues like RA synovium (63) and ulcerative colitis (64). Consequently, the increased CCR7 expression on CAPE-treated DC would not only result in enhanced migration \textit{in vitro}, but \textit{in vivo} this would also result in increased migration of these tolerogenic DC to lymph nodes or sites of inflammation, thereby respectively inducing general tolerance or dampening the immune response at the site of inflammation (5,64). This could have important implications for the use of these cells in cell-based immunotherapy of unwanted adaptive immune responses, since in patients only a very small fraction of re-in-
Uncoupling of DC maturation and CCR7 expression

jected DC traffic to secondary lymphoid organs (65) and interact with naïve T cells (reviewed in (66)). Until now the efficiency of DC migration to lymph nodes could only be increased by ex vivo transduction of DC with a CCR7 expressing adenoviral construct (67), which concomitantly induces maturation. Our data demonstrate that CCR7 expression can be uncoupled from DC maturation. Detailed knowledge of the mechanisms used by CAPE to increase functional CCR7 expression could be exploited to increase the efficiency of DC-based immunotherapies, not only for autoimmune diseases, but also for cancer treatment.

CAPE-treated DC exhibited a reduced capacity to induce both T cell proliferation and differentiation in naïve T cells. The reduced induction of T cell proliferation by CAPE-treated DC correlated with lower expression levels of co-stimulatory molecules and the maturation marker CD83 that is also involved in T cell stimulation (68). However, the impaired T cell stimulatory capacity could in part also be explained by reduced production of T cell stimulatory cytokines like IL-12 (69). The reduced Th1/Th2 polarizing capacity likely results from CAPE-induced alterations in intracellular signaling pathways, leading to changed expression of cell surface molecules and various soluble factors that are involved in the induction of T cell differentiation. Nevertheless, the decreased Th1 polarizing capacity could also be attributed to less IL-12 production and reduced expression of co-stimulatory molecules such as CD86, but also to an observed reduction in the expression of the Th1 inducing molecule ICAM-1 (70)(data not shown) and other NF-κB responsive factors (71).

In conclusion, we demonstrated that CAPE induces tolerant DC with functional increased CCR7 expression that enables them to migrate to lymph nodes or inflamed tissues. In addition, interaction of CAPE-treated DC with T cells results in less proliferation and differentiation into Th1/2 (effector) cells. Our results indicate that NF-κB inhibition in DC may provide a tool to change unwanted cellular immune responses in auto-immunity, allergy or after transplantation.
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Selective inhibition of NF-κB in dendritic cells by the NEMO-binding domain peptide blocks maturation and prevents T cell proliferation and polarization

Sander W. Tas¹, Esther C. de Jong², Najat Hajji¹, Michael J. May³, Sankar Ghosh⁴, Margriet J. Vervoordeldonk¹ and Paul P. Tak¹

¹Div. of Clinical Immunology and Rheumatology and ²Dept. of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands. ³School of Veterinary Medicine, Dept. of Animal Biology, University of Pennsylvania, Philadelphia, PA. ⁴Immunobiology Section, Yale University Medical School, New Haven, CT.

ABSTRACT

Dendritic cells (DC) are the only antigen-presenting cells (APC) for naive T cells and, therefore, they are crucial players in the initiation of immune responses. Because DC maturation and cytokine production are NF-κB dependent, we hypothesized that blocking NF-κB activity in DC by selectively targeting the inhibitor of κB (IκB) kinase (IKK) complex using the novel NF-κB inhibitor NEMO binding domain (NBD) peptide could inhibit DC maturation and other functional characteristics, resulting in modulation of the immune response. We used human monocyte-derived DC to test the biological effects of the NBD peptide in vitro. NF-κB inhibition by the NBD peptide resulted in blockade of IKK mediated IκBα phosphorylation and subsequent nuclear translocation and DNA binding of NF-κB p65 in DC. In addition, IL-6, IL-12, and TNFα production was dose-dependently blocked and NBD peptide treatment also led to a strong reduction of LPS-induced maturation. Functional analysis of these DC showed marked inhibition of T cell proliferation in the allogeneic mixed lymphocyte reaction (MLR), accompanied by less Th1 and Th2 polarization. The current study reveals for the first time the unique properties of this novel, highly specific NF-κB inhibitor in DC. Also, these data indicate that the NBD peptide could be used as an elegant tool in DC based immunotherapy for unwanted cellular immune responses.
INTRODUCTION

DC are crucial regulators of the immune response to foreign and self antigens with many unique and powerful features (reviewed in (1,2)). Through their exclusive ability to instruct naive T cells they are capable of inducing both T cell proliferation as well as anergy in response to antigens (3). The fate of naive T helper cells is determined by three signals. The first signal results from ligation of TCR by antigens presented by MHC class II on DC, which determines the antigen specificity of the response. In addition to the first signal T cell co-stimulation is required, mainly mediated by triggering of CD28 on the T cell by CD80 and CD86 expressed on the DC. Thirdly, T cell polarizing signals mediated by various soluble or membrane-bound factors are necessary. These T cell polarizing signals induce either Th1 or Th2 differentiation or regulatory T cells (reviewed in (4)).

It is well established that the transcription factor NF-κB has a key role in mediating inflammatory responses (5), including the regulation of DC development, maturation and APC function (6). NF-κB activation controls both the expression of co-stimulatory molecules and MHC, as well as the production of cytokines like IL-12 (7). Therefore, NF-κB has a major influence on all three signals that are involved in T cell activation. Consequently, inhibition of NF-κB activity results in a reduced capacity of DC to secrete pro-inflammatory cytokines and to stimulate naive CD4+ and CD8+ T cells (reviewed in (8)). This information could potentially be applied to utilize DC for changing unwanted cellular immune responses in auto-immunity, allergy or after transplantation.

The NF-κB/Rel family consists of homo- or heterodimers of the structurally related proteins RelA (p65), NF-κB1 (p50; p105), NF-κB2 (p52; p100), c-Rel and RelB. The main activated form of NF-κB is a heterodimer, consisting of p50 and the transactivating subunit p65. In most resting cells, NF-κB resides in the cytosol complexed with its inhibitor IκBα. Phosphorylation of IκBα by the IκB kinase (IKK) complex and subsequent polyubiquitination targets IκBα for degradation, releasing NF-κB dimers from the NF-κB-IκBα complex, followed by translocation to the nucleus and binding to κB enhancer elements of target genes (reviewed in (9)). The IKK complex contains two catalytic subunits, named IKKα and IKKβ, and a regulatory subunit named NEMO (NF-κB essential modulator) (10). Most signaling pathways that activate NF-κB, including those initiated by pro-inflammatory cytokines and ligation of TLR, converge at the IKK complex (9). Consequently, selective inhibition of the IKK complex has emerged as a promising strategy to block aberrant NF-κB activity in autoimmune and inflammatory diseases as well as certain cancers (11).

NF-κB activation in response to pro-inflammatory signals is dependent upon NEMO and IKKβ (12). We previously demonstrated that these subunits interact via a six amino acid sequence present in IKKβ that we named the NEMO binding domain (NBD). We further demonstrated
that a peptide spanning this domain disrupted the NEMO:IKKβ interaction in vitro and that a cell-permeable version of the NBD blocked signal-induced NF-κB activation in both cells and animal models of inflammation (13). As the NBD peptide is a highly selective inhibitor of the NF-κB pathway we sought to determine its effects on DC biology and subsequent T cell activation. Throughout our study we used human monocyte-derived DC, as the level of constitutive NF-κB activity in these cells is low and can be rapidly induced and upregulated following LPS stimulation (14).

We demonstrate that specific inhibition of NF-κB by the NBD peptide arrests DC in an immature state despite stimulation with LPS. Treatment of DC with the NBD peptide leads to reduced T cell proliferative capacity in the MLR and reduced Th1/Th2 polarization of naive T cells. DC that instructed naive T cells to become hyporesponsive were characterized by lower expression of the Th1 and Th2 polarization inducing signal transduction molecules interferon-regulatory factor (IRF) 3 and STAT6. Our results indicate that selective NF-κB inhibition in DC by the NBD peptide may provide a tool to dampen the unwanted cellular immune responses seen after transplantation or in auto-immune diseases like rheumatoid arthritis (RA), multiple sclerosis and inflammatory bowel disease.

MATERIAL & METHODS

Antibodies, cytokines, and reagents

Human IL-4 (20 x 10^8 U/mg) was obtained from Strathmann Biotec AG (Hannover, Germany). Human rGM-CSF (sp. act. 1.11 x 10^7 U/mg) was a gift of Schering-Plough (Uden, The Netherlands). Mouse mAbs to human CD28 (CLB-CD28/1) and human CD3 (CLB-T3/4E-1XE) were obtained from Sanquin (Amsterdam, The Netherlands). Mouse mAbs to phosphorylated (ph) IκBα, ph-p38 and phSTAT6 were obtained from Cell Signaling Technology (Beverly, MA). Mouse mAb to STAT6 was obtained from BD Biosciences (Franklin Lanes, NJ) and polyclonal rabbit anti-human IRF3 and anti-p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

NBD peptides

Small scale Fmoc synthesis of the peptides was carried out on a Rainin Symphony Instrument at the HHMI Biopolymer-Keck Foundation Biotechnology Resource Laboratory at Yale University. Peptides were characterized by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and analytical reverse phase HPLC analysis. The peptides were subsequently dissolved in DMSO to stocks of between 20-50 mM. The sequences of the wild-type and mutant (MUT) NBD peptides have been described previously (13). The NBD peptide contains the region of IKKβ from T735 to E745 synthesized in tandem with a membrane
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permeabilization sequence from the drosophila antennapedia homeodomain protein. The MUT peptide is identical except that W739 and W741 are replaced by alanines to render it biologically inactive.

In vitro generation and maturation of DC from peripheral blood monocytes

Venous blood from healthy donors was collected in sodium-heparin-containing tubes (BD Vacutainer Systems, Plymouth, UK). PBMC were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Torshov, Norway). Subsequently, PBMC were centrifuged on a Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient to isolate the monocytes, as previously described (15). Monocytes were washed and seeded in 24-well culture plates (Costar, Cambridge, MA) at a density of 5 x 10^5 cells/well or 6-well culture plates (Costar) at a density of 2 x 10^6 cells/well. After 60 min of incubation at 37°C, nonadherent cells were removed and adherent cells were cultured in IMDM (Life Technologies, Paisley, U.K.) containing gentamicin (86 µg/ml; Duchefa, Haarlem, The Netherlands) and 1% FCS (HyClone Laboratories, Logan, UT), supplemented with rGM-CSF (500 U/ml) and rIL-4 (250 U/ml) to obtain immature DC as described elsewhere (16). At day 3, the culture medium, including the supplements, was refreshed. On day 6, maturation of immature DC was induced by the addition of LPS (100 ng/ml; Sigma, Zwijndrecht, The Netherlands). After 48 h, full maturation into CD1a⁺CD83⁺ mature effector DC was confirmed by flow cytometric analysis.

NF-κB inhibition

Immature DC were incubated for 2 h with the indicated concentrations of the NBD peptide or controls (MUT/medium) prior to induction of maturation or stimulation by LPS. All subsequent tests were performed after harvesting and extensive washing of the cells to remove all factors.

Western blotting

After the indicated times of incubation and stimulation cells were washed twice with ice-cold PBS to remove all serum proteins and then lysed in 1x SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE on a 10% gel, using Rainbow-colored protein molecular weight markers (Amersham, Little Chalfont, U.K.) as a reference, and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in TBS containing 2% non-fat dry milk (Bio-Rad, Hercules, CA), Na₂VO₄ (2mM) and 0.05% Tween 20 during 1 h. Detection of phosphorylated and unphosphorylated proteins was performed by incubating the membranes with a primary antibody against the protein of interest overnight at 4°C. The membranes were subsequently washed and incubated with the appropriate secondary Ab in TBS containing 2% non-fat dry milk, Na₂VO₄ (2mM) and 0.05% Tween 20 for 1 h at room temperature, and after extensive washing assayed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfront, UK). Densitometry was performed using GeneTools software (SynGene, Cambridge, UK).
Immunofluorescence staining of p65 and HLA-DR and confocal microscopy analysis

Immature DC were seeded onto LAB-TEK glass chamber slides (Nalgene Nunc International, Naperville, IL) at a density of 500 cells per well and stimulated for 4 h with LPS in the presence or absence of NBD peptides (50 μM) or controls. Subsequently, cells were washed, air-dried and fixed in cold acetone for 10 min. The slides were then washed extensively with PBS and incubated with optimal dilutions of the primary Ab in PBS containing 1% FCS and 1% human serum for 60 min at room temperature, followed by 60 min incubation at room temperature with Alexa 488-conjugated goat-anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) and Cy3-conjugated streptavidin (Sigma) in PBS containing 1% FCS and 1% human serum to detect p65 and HLA-DR, respectively. For each fluorochrome label, negative control antibodies were included. Finally, the slides were incubated with Hoechst for 5 min to label the nuclei and mounted in Vectashield (Vector Labs, Burlingame, CA) for confocal microscopy analysis. RelA/p65 and HLA-DR were visualized using a Leica TCS SP (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr/HeNe laser combination. Images were taken using a ×40 1.25 NA objective.

Nuclear cell extract preparation and NF-κB DNA binding activity

Nuclear cell extracts were prepared by employing a kit from Active Motif (Rixensart, Belgium). In brief, cells were washed, collected in ice-cold PBS in the presence of phosphatase inhibitors and centrifuged at 500rpm for 5 min. The pellets were resuspended in a hypotonic buffer, treated with detergent and centrifuged at 14,000g for 30 s. After collection of the cytoplasmic fraction, the nuclei were lysed and nuclear proteins solubilized in lysis buffer containing proteasome inhibitors. The binding of NF-κB to DNA was measured in nuclear extracts with a specific chemiluminescence-based TransAM™ NF-κB p65 assay kit (Active Motif), according to the manufacturer’s instructions. This assay uses multi-well plates coated with an unlabeled oligonucleotide containing the consensus binding site for NF-κB (5'-GGGACTTTCC-3') (17). Nuclear proteins (10 μg) were added to each well and incubated for 1 h to allow NF-κB DNA binding. Subsequently, activated p65 DNA binding was quantified by antibody detection, followed by spectrophotometry.

Cytokine production by DC

DC (2 x 10⁴ cells/well) were stimulated with CD40ligand (CD40L)-expressing mouse plasmacytoma cells (J558 cells, 2 x 10⁴ cells/well; a kind gift from Dr. P. Lane, University of Birmingham, Birmingham, U.K.), in the presence of rIFN-γ (1000 U/ml, U-Cytech, Utrecht, The Netherlands), in 96-well flat-bottom culture plates (Corning Life Sciences, Schiphol-Rijk, The Netherlands) in IMDM containing 10% FCS in a final volume of 200 μl. Supernatants were harvested after 24 h and stored at -20°C until the levels of IL-12p70, TNFα and IL-6 secretion were measured by specific solid-phase sandwich ELISA as described previously (18). The detection limits are as follows: IL-12p70, 3 pg/ml; TNFα 10 pg/ml; IL-6, 20 pg/ml.
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Analysis of cell surface molecule expression by flow cytometry
At day 8, the maturated “effector” DC were analyzed for the expression of cell surface molecules by flow cytometry. Mouse anti-human mAbs against the following molecules were used: CD1a (OKT6; Ortho Diagnostic System, Beerse, Belgium), CD83 (HB15a, IgG2b; Immunotech, Marseille, France), CD86 (IT2.2, Pharmingen, Erembodegem, Belgium), and HLA-DR (L234, IgG2a; BD Biosciences, San Jose, CA). Primary mAb were detected by FITC-conjugated goat anti-mouse mAb (Jackson ImmunoResearch Laboratories, West Grove, PA).

Mixed lymphocyte reaction
Effector DC were also tested for their ability to stimulate allogeneic naive Th cells in a MLR. Highly purified CD4⁺CD45RA⁺CD45RO⁻ naive Th cells (>98% as assessed by flow cytometry) were isolated from PBMCs or PBLs using a CD4⁺ T cell isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), followed by depletion of CD4⁺CD45RO⁺ T cells using PE-labeled anti-CD45RO (Dako, Glostrup, Denmark) and anti-PE-beads (Miltenyi Biotec). Naive Th cells (2.5 x 10⁴ cells per 200 μl) were cocultured in 96-well flat-bottomed culture plates with different concentrations of mature DC. After 5 d, cell proliferation was assessed by the incorporation of [³H]thymidine (Radiochemical Center, Amersham, Little Chalfont, U.K.) after a pulse with 13 kBq per well during the last 16 h, as measured by liquid scintillation counting.

Determination of naive Th cell polarization by DCs
Highly purified CD4⁺CD45RA⁺CD45RO⁻ naive Th cells (2 x 10⁴ cells/200 μl of IMDM with 10% FCS) were cocultured with 5 x 10⁵ effector DCs coated with *Staphylococcus* enterotoxin B (Sigma-Aldrich; final concentration, 10 pg/ml) in 96-well flat-bottom culture plates (Costar). At day 5, human rIL-2 (10 U/ml, Cetus, Emeryville, CA) was added, and the cultures were expanded for the next 7 days. On day 12, the quiescent Th cells were counted and equal cell numbers were restimulated with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 6 h. During the last 5 h Brefeldin A (10 μg/ml; Sigma-Aldrich) was present to inhibit secretion of intracellularly produced proteins. Single cell IL-4 and IFN-γ production was determined by intracellular flow cytometric analysis. Cells were fixed in 2% formalin (Merck, Darmstadt, Germany), permeabilized with 0.5% saponin (Sigma) and stained with anti-human IFN-γ-FITC and anti-human IL-4-PE (both from BD Biosciences, Franklin Lakes, NJ). The cells were evaluated by FACScalibur (BD Biosciences).

Statistical analysis
Data were analyzed for statistical significance (GraphPad, InStat, version 2.02) using ANOVA or Student’s t test. A p value <0.05 was taken as the level of significance.
RESULTS

NBD peptide specifically blocks IκBα phosphorylation in DC

Since the NBD peptide has been demonstrated to specifically inhibit IκBα phosphorylation by IKK in a number of cell types (13), we investigated the potential of these unique peptides to do so in DC. Therefore, the phosphorylation status of IκBα in cytoplasmic extracts from DC was determined by Western blot using Abs specific for the phosphorylated form of this protein. Induction of IκBα phosphorylation was readily detected in cytoplasmic extracts prepared from DC stimulated with LPS compared to unstimulated DC (Figure 1A). Pretreatment with NBD peptides for 2 h significantly inhibited phosphorylation of IκBα following LPS stimulation, whereas the control peptide MUT did not have any inhibitory effects. After 30 min of LPS stimulation NBD pretreatment potently inhibited phosphorylation of IκBα and reduced the level of phospho-IκBα to the levels observed in unstimulated cells. Similar results were obtained when DC were stimulated with IL-1β (10 ng/ml) or TNFα (10 ng/ml) for 30 min (data not shown). To demonstrate that the inhibition of IκBα phosphorylation is specific for the NF-κB pathway, we evaluated the effects of the NBD peptide on MAPK kinases by investigating p38 activation. The phosphorylation status of p38 was determined in the same cytoplasmic extracts by Western blot using Abs specific for the phosphorylated form of this protein. LPS stimulation of DC resulted in an increase in p38 phosphorylation that was not blocked by the NBD or MUT peptides (Figure 1B). Taken together, these results demonstrate that the NBD peptide specifically inhibits IκBα phosphorylation by blocking the NF-κB pathway restricted kinase IKK in DC, while not affecting phosphorylation induced activation of p38 by MAPK kinases.

NBD peptide inhibits nuclear translocation and DNA binding activity of NF-κB.

Since a block in IKK activity and IκBα phosphorylation should consequently result in sequestration of NF-κB in the cytoplasm and reduced nuclear translocation, the effect of NBD treatment on nuclear translocation of the main transactivating NF-κB subunit, p65, was studied in DC. Confocal microscopic analysis of DC that were stimulated with LPS for 4 h showed unambiguous translocation of p65 to the nucleus (Figure 2). Of importance, NBD pretreatment of these DC resulted in a complete block in nuclear translocation of p65 in all cells. The MUT peptide had no inhibitory effect on p65 translocation. In addition, HLA-DR expression that has been known to be controlled by NF-κB responsive genes, was also reduced in NBD treated DC (Figure 2). Subsequently, nuclear fractions were prepared to investigate the DNA binding capacity of p65 in these DC. LPS-induced NF-κB p65 DNA binding was reduced more than 2-fold upon NBD pretreatment for 2 h (OD 2.06 ± 0.05 vs. 0.92 ± 0.04; p<0.01), whereas the MUT peptide had no effect on DNA binding (Figure 3). Background NF-κB p65 DNA binding of unstimulated DC was substracted from the values listed above. These results provide
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... evidence that NBD pretreatment of DC blocks nuclear translocation and DNA binding of NF-κB in response to LPS.

Pro-inflammatory cytokine production by DC is dose-dependently inhibited by the NBD peptide

Next, we investigated whether NF-κB inhibition by NBD peptide treatment may have consequences for the production of cytokines that are important for T cell responses. Therefore, DC pre-treated with increasing concentrations of NBD or MUT peptides were stimulated with LPS for 2 days. Subsequently, DC were stimulated with CD40L for 24 h to determine IL-6, IL-12p70 and TNFα production in the supernatant. NBD treated DC exhibited reduced levels of IL-6, IL-12p70 and TNFα secretion after re-stimulation, in a dose dependent manner (Figure 4). NBD concentrations of 200 μM resulted in maximal cytokine suppression, whereas equal concentrations of the MUT peptide did not have an inhibitory effect on cytokine production. Similar results were obtained with immature DC incubated with the NBD peptide and stimulated with CD40L to induce cytokine production. No significant differences in DC viability were observed between treatment and control groups based on flow cytometry (data not shown). LPS-induced maturation in the presence of NBD peptides results in a reduced capacity of effector DC to produce T cell instructing cytokines upon restimulation.

NBD peptide blocks LPS-induced upregulation of MHC class II and co-stimulatory molecules

The observed reduction in cytokine production after NBD treatment suggests that NF-κB inhibition in LPS stimulated DC results in an altered phenotype. In addition to pro-inflammatory cytokine production, the expression of co-stimulatory molecules and antigen presenting molecules like MHC class II is also transcriptionally regulated by NF-κB. Hence, we examined whether pre-treatment with NBD affected the up-regulation of these molecules after LPS-induced DC activation. Immature DC were pre-treated with NBD or MUT peptides for 2 h followed by LPS stimulation for 2 days. Cells were then stained and analyzed by flow cytometry for the presence of surface HLA-DR, CD83, and CD86. We found that the NBD pep-
tide abrogated the up-regulation of HLA-DR ($p<0.05$), CD83 ($p<0.05$), and CD86 ($p<0.05$) after LPS stimulation (Figure 5; upper panels). This NBD mediated inhibition was dose dependent in all experiments (data not shown). In contrast, the MUT peptide did not influence LPS-induced up-regulation of HLA-DR, CD83, and CD86 (Figure 5; lower panels). The reduced surface expression of HLA-DR on NBD treated DC as quantified by FACS analysis confirmed the earlier findings by confocal microscopy (see Figure 2). These data show that NF-κB inhibition by the NBD peptide alters the maturation state and co-stimulatory molecule expression of LPS stimulated DC, which is likely to have major consequences for the capacity of these DC to present antigens to naive T cells.

The NBD peptide inhibits the capacity of DC to induce T cell proliferation

To examine the potential of NBD treated DC to prime naive T cells, immature DC were pretreated with NBD peptides, stimulated with LPS for 2 days and mixed with allogeneic naive CD4$^+$ T cells. T cell proliferation was measured by $[^{3}H]$thymidine incorporation. We found that NBD treatment of DC results in dramatically reduced capacity to induce T cell proliferation.

Figure 2. NBD peptide blocks nuclear translocation of p65 and MHC class II surface expression. See Color figures

Series of confocal images of monocyte-derived DC cultured on glass chamber slides and stimulated for 4 h with LPS (200 ng/ml) in the presence or absence of NBD/MUT peptides (50 μM) or controls. Cells were fixed in cold acetone and stained for p65 and HLA-DR expression as described in the Methods section. Nuclei were stained with Hoechst and cells were analyzed by scanning the entire cell using a confocal laser microscope. In the overlay picture nuclear translocation can be evaluated. Representative pictures from one experiment are shown. Results are representative for three independent experiments.
Selective NF-κB inhibition in DC blocks maturation and T cell proliferation/polarization compared to MUT treated and control DC at all DC:T cell ratios (Figure 6). Direct effects of the NBD peptide on T cell proliferation were excluded by extensive washing in the experiments. These data are in agreement with the observed reduced production of the cytokines IL-6, IL-12p70 and TNFα, and the inhibited upregulation of surface molecules such as HLA-DR, CD80 and CD86 that are essential for effective induction of T cell proliferation.

Reduced Th polarization of naive T cells by NBD treated DC
Because T cell proliferation was significantly reduced in the MLR, we further investigated which type of immune response is induced by NBD peptide treated DC. Hence, we loaded NBD treated DC with the superantigen *Staphylococcus* enterotoxin B and cocultured them

![Figure 3. NBD peptide blocks DNA binding of NF-κB.](image)
Immature DC were stimulated for 4h with LPS (200 ng/ml) in the presence or absence of NBD/MUT peptides (50 μM) or controls. Nuclear cell extracts were prepared and the binding of NF-κB p65 to DNA was quantified by an ELISA-based kit. Specific inhibition of NF-κB by the NBD peptide resulted in significantly decreased p65 DNA binding (*p<0.05). The results are expressed as mean optical density (OD) at 450 nm ± SD of replicate determinations. The data shown are from one of two independent experiments that yielded similar results.

![Figure 4. Pro-inflammatory cytokine production by DC is dose-dependently inhibited by the NBD peptide.](image)
Immature DC were incubated with the indicated concentrations of NBD peptides or controls and matured for 2 days with LPS (100 ng/ml). After 48 h, the cells were thoroughly washed and stimulated with the CD40L-expressing mouse fibroblast cell line J558. Supernatants were harvested after 24 h and secreted cytokines were measured by ELISA. A, IL-6; B, IL-12p70; C, TNFα. Results are expressed as mean ± SD from one representative experiment of three performed in triplicate. *, p < 0.05; **, p < 0.001, compared to MUT and control.
with naive Th cells. After 12 days we re-stimulated the T cells and single cell IL-4 and IFN-γ production was measured by intracellular flow cytometric analysis to determine the percentage of IL-4 and IFN-γ producing T cells after DC instruction. NBD peptide mediated NF-κB blockade in *Staphylococcus enterotoxin* B-primed DC resulted in a markedly reduced development of both IFN-γ (5.16% vs. 15.45% in control samples; *p*<0.05) and IL-4 (2.65% vs. 16.89% in control samples; *p*<0.01) producing Th cells from naive precursors, whereas no difference in the development of IL-4 or IFN-γ producing Th cells was observed when MUT or control DC were used to stimulate naive Th cells (Figure 7A). Also in this naive T cell outgrowth experiment, proliferation was significantly reduced in the T cells that had encountered NBD treated DC compared to MUT and control DC (Figure 7B; *p*<0.05 NBD vs. MUT/Control). These data show that in addition to reduced T cell proliferation, NBD treatment of LPS stimulated DC also blocks Th cell polarization by inhibiting both Th1 and Th2 differentiation, resulting in a non-polarized Th phenotype.

NBD treatment results in reduced activation of Th1- and Th2-induction associated signaling molecules in DC

Since NBD treated DC exhibited decreased capacity to induce Th1 and Th2 differentiation of naive T cells, we explored whether as a consequence of NF-κB inhibition by the NBD peptides other signaling molecules that are involved in the Th polarizing capacity of DC, like members of the TRIF-related adapter molecule (TRAM)/TIR-domain-containing adapter-inducing interferon-beta (TRIF)/IRF and JAK/STAT family, were also affected (4,19). Gel electrophoresis and

**Figure 5.** NBD peptide blocks LPS-induced up-regulation of MHC class II and co-stimulatory molecules.

Immature DC were cultured with LPS (100 ng/ml) in the presence or absence of NBD peptides (50 μM). After 2 days, DC were analyzed for the expression of cell surface molecules HLA-DR, CD83 and CD86 by flow cytometry. The filled histograms represent the specific expression of surface markers on DC incubated with NBD (*upper panels*) or MUT peptides (*lower panels*), and the open histograms represent control DC that were LPS stimulated without the addition of peptides. Data are representative of at least five independent experiments.
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subsequent Western blot analysis using cell lysates of LPS stimulated DC showed reduced levels of IRF3 in NBD treated cells (Figure 8A), which was accompanied by decreased production of the IRF3-dependent chemokine IFN-γ inducible protein 10 in these DC (data not shown). The association between IRF3 expression in DC and the induction of Th1 differentiation is paralleled by the association between STAT6 phosphorylation in DC and Th2 induction (20,21). Recently, it was demonstrated that IL-4 induced STAT6 activation could be negatively influenced by NF-κB inhibition (22). Although generation of immature monocyte-derived DC requires IL-4, this cytokine is not present in the culture during LPS-induced maturation in the

Figure 6. The NBD peptide inhibits the capacity of DC to induce T cell proliferation in the allogeneic MLR. Immature DC were incubated with NBD peptides (50 μM) or controls and matured for 2 days with LPS (100 ng/ml). After 48 h, the cells were thoroughly washed and different concentrations of DC were used to stimulate CD4+ Th cells in the presence of 1 ng/ml SEB. The proliferative response was determined at day 5 of coculture by [3H]TdR incorporation. Data are presented as mean cpm ± SD of triplicate cultures. Results are representative of three independent experiments.

Figure 7. Incubation of DC with NBD peptides blocks proliferation and subsequent Th1/2 differentiation from human naive Th cells. (A) Naive T cells that encounter NBD treated DC exhibit less Th1/Th2 polarization in the naive T cell outgrowth assay. CD4+CD45RA+ naive Th cells (2 x 10⁴) were stimulated with superantigen Staphylococcus aureus enterotoxin B (10 pg/ml), presented by DC (5 x 10³ cells) that were earlier matured in the absence or presence of NBD peptides with LPS (100 ng/ml). After 12 days, the responder Th cells had become quiescent and were restimulated with PMA/ionomycin for 6 h, the last 5 h in the presence of brefeldin A. The percentages of IFN-γ and IL-4 producing cells were measured by intracellular FACS analysis. *, p<0.05; **, p<0.01. (B) Naive T cells that encounter NBD treated DC also exhibit less proliferation in the naive T cell outgrowth assay. *, p<0.05. Results are presented as mean percentage positive cells ± SD from one representative experiment out of three, performed in duplicates.
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presence or absence of NBD peptides. Therefore, we investigated whether NF-κB inhibition by the NBD peptide affected STAT6 phosphorylation. We found that treatment with the NBD peptide partially reversed STAT6 phosphorylation in DC, while not affecting basal levels of STAT6 (Figure 8B), to some extent explaining the observed decrease in the induction of Th2 differentiation by NBD treated DC.

DISCUSSION

The current study reveals for the first time the unique properties of the NBD peptide to mediate immunoregulation in DC. The NBD peptide selectively inhibited the NF-κB pathway at the level of the IKK complex, resulting in reduced IκBα phosphorylation and a block in subsequent nuclear translocation and DNA binding of p65. NF-κB activity was inhibited without affecting MAPK kinase mediated p38 phosphorylation in DC, which is consistent with the observation in other cell types that the NBD peptide does not affect activation of MAPK signaling proteins (13,23). Pretreatment with the NBD peptide effectively suppressed the secretion of pro-inflammatory cytokines by DC, as well as the increase of co-stimulatory molecules and maturation markers following stimulation with LPS. In addition, NBD treated DC exhibited a reduced capacity to stimulate naive CD4+ Th cells, that also showed less Th1 or Th2 polarization.
The NBD peptide is a highly specific inhibitor of IKK activity since it consists of a small fragment of IKKβ fused with a membrane translocation sequence (13). Recently, it was reported that in addition to phosphorylation of IκBα, phosphorylation of Ser536 located in the trans-activation domain of p65 is also dependent on IKK activity (24). Therefore, NBD mediated suppression of IKK may directly reduce the transcriptional function of p65 containing NF-κB complexes. Because the NBD peptide impaired both nuclear translocation of p65, demonstrated by retention of NF-κB complexes in the cytoplasm, and DNA binding of this subunit, this could be an additional mechanism by which NF-κB activation is blocked.

Blockade of NF-κB activation in DC can lead to altered antigen presentation resulting in the induction of T cell tolerance (reviewed in (8)). The pleiotropic cytokine IL-10 is also known to have regulatory effects on DC resulting in the induction of T cell anergy (25,26). Recently, it was revealed that IL-10 exerts its immunomodulatory effect on DC via suppression of IKK activity (27). The results obtained in that study largely resemble the biologic consequences of the NBD peptide on DC. In line with this, addition of the NBD peptides at the initiation of culture resulted in the generation of macrophage-like cells (data not shown), suggesting earlier observations that IL-10 blocks differentiation and maturation of DC (25) are probably the result of IKK inhibition. Although IL-10 mediated suppression of IKK activation in DC was effective, it was only accomplished after 12 to 24 hr pretreatment (26,27), whereas the NBD peptide blocks IKK activity already within 2 hours. Furthermore, IL-10 is also known to have certain immunostimulatory effects (reviewed in (28)). Taken together, these findings indicate that direct targeting of IKK in DC is perhaps more suitable for tolerance induction as a therapeutic strategy than immunomodulation via IL-10.

Literature on the role of the IKK complex in LPS induced NF-κB activation in DC is not conclusive. Several studies suggest that IKK is pivotal for LPS induced NF-κB activation in DC (29,30). However, a recent study using an adenoviral vector encoding a dominant negative IKKβ protein reported IKK independent NF-κB activation in DC by LPS (31). Based on the data presented here, we conclude that LPS induced maturation of DC is IKK dependent, because treatment with the highly specific IKK inhibitor NBD resulted in markedly reduced IκBα phosphorylation and blocked nuclear translocation of p65. Also, the production of NF-κB responsive pro-inflammatory cytokines and expression levels of co-stimulatory molecules and other surface markers were reduced.

When examining the interaction of NBD treated DC with naive T cells, both reduced T cell proliferation and differentiation were observed. The reduced induction of T cell proliferation by NBD treated DC correlated with lower expression levels of the co-stimulatory molecule CD86 as well as the surface maturation marker CD83 that is also involved in T cell stimulation (32). The reduced Th1/Th2 polarizing capacity of NBD treated DC depends on altered
intracellular signaling pathways leading to changed expression of cell surface molecules and various soluble factors. The observed decreased Th1 polarizing capacity can for instance be attributed in part to reduced IL-12 production and reduced expression of surface co-stimulatory molecules such as CD86, but also to an observed reduction in the expression of the Th1 inducing molecule ICAM-1 (data not shown). The complex consequences of IKK blockade on intracellular signal transduction pathways other than NF-κB may also influence the Th polarizing capacity of these DC. Triggering of TLR4 by LPS has been demonstrated to result in the interaction of TRAM with TRIF leading to the activation of IRF3 (33). IRF3 then induces primary activation of type I interferons like IFN-β and the chemokine IFN-γ inducible protein 10, promoting Th1 differentiation of CD4+ T cells by activating STAT4 (34,35). The negative effect of the NBD peptide on IRF3 signaling was paralleled by reduced production of IFN-γ inducible protein 10 (data not shown), and is in agreement with recent data showing that the poxvirus protein N1L inhibits toll-like receptor induced NF-κB and IRF3 signaling by targeting IKK (36). Taken together, we have shown that IKK inhibition via the NBD peptide has inhibitory effects on IRF3 signaling resulting in reduced Th1 polarizing capacity.

Analogous to IRF3 associated Th1 induction, STAT6 is a key signal transduction molecule that is involved in the induction of Th2 polarization by DC (21,37). The observed inhibitory effects of the NBD peptide on STAT6 phosphorylation are consistent with a recent study showing significantly reduced binding of STAT6 and NF-κB oligonucleotides to lung nuclear proteins corresponding with reduced expression of Th2 cytokines after inhalation of activated protein C in a murine asthma model (38). Our data could indicate that the direct association and synergistic activation of the NF-κB and STAT signaling pathways (39) may also act reciprocally in such a way that NF-κB activity is required for full STAT activation. Consequently, NF-κB inhibition in DC reduces STAT6 phosphorylation, which to some extent explains the observed decrease in the induction of Th2 differentiation. When extrapolating the effects of the interaction between NBD treated DC and T cells to the in vivo situation, this suggests that immunotherapy with NBD treated DC might be able to prevent the unwanted immune response observed after transplantation, and to suppress T cell activation and polarization in autoimmunity.

Compared to glucocorticoids (40,41) and other relatively non-specific pharmacological NF-κB inhibitors (30,41), the NBD peptide is far more specific and, therefore, may have less side-effects when administered in vivo. As steroids are one of the most powerful drugs in the treatment of auto-immune diseases like RA (42), it is to be expected that NF-κB inhibition via the NBD peptide could result in an even better clinical effect without adverse side-effects such as osteoporosis. The therapeutic potential of the NBD peptide has been explored in two experimental mouse models of acute inflammation (13), as well as a mouse model of arthritis (23) and multiple sclerosis (43), resulting in significantly reduced inflammation. At
least part of these therapeutic effects could be due to NBD mediated NF-κB inhibition in DC, with reduced immunostimulation or even immunoregulation as a result.

In summary, our work clearly demonstrates that the NBD peptide effectively immunoregulates the APC function of DC through suppression of IKK activity and subsequent NF-κB activation, resulting in reduced T cell responses. Inhibition of the T cell stimulatory and Th1/2 inducing capacity of NBD treated DC is in part the consequence of the effects that IKK inhibition has on IRF3 and STAT6 activation. The fact that IKK also governs NF-κB activation in many other cells involved in the inflammatory process (12), supports the notion that NBD treatment could have a beneficial effect in a variety of disorders, including RA, chronic inflammatory bowel disease and transplant rejection.
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Chapter 10

Induction of indoleamine 2,3-dioxygenase (IDO) in dendritic cells requires non-canonical NF-κB signaling

Sander W. Tas¹, Margriet J. Vervoordeldonk¹, Najat Hajji¹, Joost H. N. Schuitemaker², Michael J. May³, Sankar Ghosh⁴, Martien L. Kapsenberg², Paul P. Tak¹ and Esther C. de Jong²

¹Div. of Clinical Immunology and Rheumatology, and ²Dept. of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands. ³School of Veterinary Medicine, Dept. of Animal Biology, University of Pennsylvania, Philadelphia, PA. ⁴Immunobiology Section, Yale University Medical School, New Haven, CT.
ABSTRACT

Ligation of CD40 on dendritic cells (DC) induces early production of inflammatory mediators via canonical NF-κB signaling, as well as late expression of the anti-inflammatory enzyme indoleamine 2,3-dioxygenase (IDO) via unknown signal transduction. By selective blocking of either the canonical NF-κB pathway using the NEMO-binding domain peptide or the non-canonical NF-κB pathway by small interfering RNA, we demonstrate that IDO expression requires non-canonical NF-κB signaling. In addition, selective activation of the non-canonical NF-κB pathway results in non-inflammatory IDO-expressing DC that promote the development of regulatory T cells from naïve T cells. These findings reveal an important role of the non-canonical NF-κB pathway in the regulation of T cell immunity.
INTRODUCTION

Dendritic cells (DC) are key regulators of adaptive immunity by selectively promoting or suppressing T cell responses (1). One of the suppressive mechanisms involves the expression of the enzyme indoleamine 2,3-dioxygenase (IDO) by DC (2). IDO degrades the essential amino acid tryptophan into kynurenine, which leads to tryptophan depletion resulting in suppression of T cell proliferation (3–5) or induction of apoptosis in activated T cells both in vitro and in vivo (6), and, consequently, the induction of tolerance (7,8). IDO can be induced in DC by a variety of stimuli, including ligation of CD40 or CD80/CD86 by respectively CD40L (3,9,10) or CTLA-4 (11,12) on activated T cells, as well as soluble factors like IFN-γ and IL-1 (reviewed in (2)). Some other factors, like LPS, require additional signals such as IFN-γ to effectively induce IDO in DC (3,13). Remarkably, the conditions resulting in the expression of anti-inflammatory IDO also result in the expression of pro-inflammatory cytokines.

NF-κB transcription factors are essential for the expression of pro-inflammatory cytokines in DC (14) and have been implicated in IDO induction (15). NF-κB can be activated via two distinct signal transduction pathways. The canonical (also known as classical) NF-κB pathway requires activation of the IKK complex, consisting of the catalytic subunits IKKα and IKKβ, and the regulatory subunit NEMO/IKKγ, and controls NF-κB activation in response to pro-inflammatory stimuli like LPS, TNFα and CD40L (16–19). Activation of this pathway results predominantly in the activation, nuclear translocation and DNA binding of the classical NF-κB dimer p50-RelA. In this pathway IKKβ is essential for NF-κB activation, whereas IKKα is dispensable for the activation and induction of NF-κB DNA-binding activity in most cell types (19–21).

In contrast, the noncanonical (also known as alternative) pathway is strictly dependent on IKKα homodimers and does not require IKKβ nor NEMO/IKKγ (22,23). The target for IKKα homodimers is NF-κB2/p100, which upon activation of IKKα by NF-κB-inducing kinase (NIK) is incompletely degraded into p52, resulting in the release and nuclear translocation of mainly p52-RelB dimers. This pathway can be triggered by the activation of members of the TNF-receptor superfamily such as the lymphotoxin β receptor, B-cell activating factor belonging to the TNF family (BAFF)-receptor and CD40 (that also induce canonical NF-κB signaling), but not via pattern recognition receptors such as Toll-like receptor 4 (TLR4), the receptor for LPS (24).

It has been suggested that the canonical and non-canonical NF-κB pathways play distinct roles in immunity (reviewed in (25)). Recent literature proposes a role for the non-canonical pathway in the regulation of immune responses, as IKKα is implicated in the negative regulation of inflammation (26) and NIK has a role in the development of regulatory T cells (Treg)(27). However, the precise mechanisms involved have not been fully elucidated yet.
Although regulatory Treg induce IDO in DC (12), it has not been reported whether IDO-expressing DC may also give rise to Treg (reviewed in (2)). We investigated whether the induction of IDO and the induction of pro-inflammatory cytokines require different NF-κB activation pathways and hypothesized that the non-canonical NF-κB pathway is essential for the induction of regulatory functions in DC via the induction of IDO.

MATERIAL AND METHODS

Antibodies, cytokines, and reagents
Human IL-4 (20 x 10^8 U/mg) was obtained from Pharma Biotechnology (Hanover, Germany). Human rGM-CSF (sp. act. 1.11 x 10^7 U/mg) was a gift of Schering-Plough (Uden, The Netherlands). Human IL-3 (10 ng/ml) was obtained from Strathmann Biotech, Hanover, Germany. Mouse mAbs to human CD28 (CLB-CD28/1) and human CD3 (CLB-T3/4E-1XE) were obtained from Central Laboratory of The Netherlands Red Cross Blood Transfusion Center (Amsterdam, The Netherlands). Mouse mAbs to phosphorylated IκBα (phospho-IκBα) and total IκBα, and polyclonal rabbit anti-β-actin were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal rabbit anti-human RelB, anti-p65 and anti-IKKα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb to human IDO was a kind gift of Dr. O. Takikawa (Hokkaido University, Sapporo, Japan).

In vitro generation and maturation of DC from monocytes or direct isolation of BDCA1+ and BDCA4+ cells from peripheral blood
Monocyte-derived DC were obtained as described previously (28). On day 6, maturation of immature DC was induced by the addition of LPS (100 ng/ml; Sigma, Zwijndrecht, The Netherlands) or CD40 ligand (CD40L)-expressing mouse plasmacytoma cells (irradiated J558 cells, 1:1 ratio with DC; a kind gift from Dr. P. Lane, University of Birmingham, Birmingham, U.K.) in the presence or absence of 1-methyl-DL-tryptophan (MT; 50 μg/ml). After 48 h, full maturation into CD1a+CD83+ mature effector DC was confirmed by flow cytometric analysis. BDCA1+ and BDCA4+ cells were isolated from peripheral blood using specific isolation kits from Miltenyi Biotec (Bergisch Gladbach, Germany). BDCA1+ and BDCA4+ cells were cultured in the presence of GM-CSF and IL-3 respectively.

NBD peptides and NBD-mediated NF-κB inhibition
NEMO binding domain (NBD) peptides were synthesized as described previously (29) and subsequently dissolved in DMSO to stocks of 50 mM. The sequences of the wild-type and mutant (MUT) NBD peptides have been described previously (29). To study the effect of IKKβ inhibition, immature DC were incubated for 2h with the NBD peptide or controls (MUT/medium) prior to induction of maturation or stimulation by LPS/CD40L. NBD/MUT peptides
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were used at a concentration of 50 μM, unless indicated otherwise. All subsequent tests were performed after harvesting and extensive washing of the cells to remove all factors as described previously (30).

Western blotting

After the indicated times of incubation and stimulation cells were washed twice with ice-cold PBS to remove all serum proteins and then lysed in 1x SDS-PAGE sample buffer. Western blotting was performed as described previously (30). Densitometry was performed using Quantity One software (Bio-Rad, Hercules, CA).

IDO activity

IDO activity was determined according to Feng and Taylor (31). In brief, cells were harvested, lyophilized, resuspended in PBS and cleared from insoluble material by centrifugation. The supernatant was incubated with L-tryptophan in a reaction buffer for 30 min at 37°C, after which the reaction was terminated with TCA. The resulting N-formylkynurenine, was hydrolyzed to kynurenine at 50°C for 30 min, followed by the addition of an equal volume of Ehrlich reagent (Sigma Aldrich). The product was read at 490 nm in a microplate reader (Titertek multiskan MCC/340; Titertek, Huntsville, AL). OD values above background (PBS only) were used to calculate fold-induction. The basal level of expression in untreated cells was set at 1.0. IDO activities were corrected for protein content (Bio-Rad protein assay; Bio-Rad, Hercules, CA).

Immunofluorescence staining of RelB and confocal microscopy analysis

NBD or MUT pre-treated (50 μM) DC were stimulated for 4 h with LPS or CD40L, washed three times, followed by centrifugation onto glass slides (Superfrost) at a density of 500 cells per slide. Subsequently, cells were air-dried and fixed in cold acetone for 10 min. The slides were then washed extensively with PBS, and stained for RelB expression using an anti-RelB primary Ab (Santa Cruz) as described previously (30). RelB expression was visualized using a Leica TCS SP (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr/HeNe laser combination. Images were taken using a ×40 1.25 NA objective.

siRNA experiments

To date, no specific pharmacological inhibitors for IKKα exist to selectively block the non-canonical pathway of NF-κB activation (32). Therefore, we used siRNA to target this pathway. Immature DC were seeded into 24 well plates (1.5x10⁵ cells; 400 μl) in serumfree IMDM. Lipofectamine 2000 (Invitrogen, Breda, The Netherlands) was pre-diluted 4:100 in serumfree IMDM (40 μl) and stock concentrations of siRNA for NIK (siNIK), IKKα (siIKKα) or control scrambled, non-blocking RNA (C) (50 nmol/ml; Ambion, Cambridge, UK) were pre-diluted 3:100 in serumfree IMDM (40 μl) for 5 min. Subsequently, Lipofectamine 2000 and siRNA pre-dilutions were mixed and incubated for 30 min at RT. Next, this mix was slowly added to the
DC (80 μl/well) and incubated for 4 h at 37°C. After that, 120 μl IMDM containing 50% FCS was added and DC were either left unstimulated (200 μl IMDM 10% FCS) or stimulated with CD40L (irradiated J558 cells, 1:1 ratio with DC; 200 μl IMDM 10% FCS) for the indicated times. To evaluate transfection efficiency, FAM-labeled control RNA was used instead of siRNA in the method described above. After 24 h transfected DC were analyzed by flow cytometry for FAM content. The siRNA sequences used to target human mRNA sequences were as follows, siNIK: 5’-GCUCCGUCUACAAGCUUGAtt-3’ (sense) and siIKKα: 5’-GGCCUGUGAUGUCCUGAAtt-3’ (sense).

Cytokine production by DC
DC (2 x 10^4 cells/well) were stimulated with CD40 ligand (CD40L)-expressing mouse plasmacytoma cells (J558 cells, 2 x 10^4 cells/well; a kind gift from Dr. P. Lane, University of Birmingham, Birmingham, U.K.), in 96-well flat-bottom culture plates (Corning Life Sciences, Schiphol-Rijk, The Netherlands) in IMDM containing 10% FCS in a final volume of 200 μl. Supernatants were harvested after 24 h and stored at -20°C until the levels of IL-12p70 or IL-6 were measured by specific solid-phase sandwich ELISA as described previously (33).

Mixed lymphocyte reactions
Highly purified CD4+CD45RA+CD45RO- naive Th cells (>98% as assessed by flow cytometry) were isolated from PBMCs using a CD4+ T cell isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), followed by depletion of CD4+CD45RO+ T cells using PE-labeled anti-CD45RO (Dako, Glostrup, Denmark) and anti-PE-beads (Miltenyi Biotec). Naive Th cells (2.5 x 10^4 cells per 200 μl) were cocultured in 96-well flat-bottomed culture plates with different concentrations of mature DC. After 5 d, cell proliferation was assessed by the incorporation of [H]thymidine (Radiochemical Center, Amersham, Little Chalfont, U.K.) after a pulse with 13 kBq per well during the last 16 h, as measured by liquid scintillation counting. In other experiments, 5 x 10^4 anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) pre-activated total CD4+ T cells were co-cultured with 1 x 10^4 effector DC in the presence or absence of 1-methyl-DL-tryptophan (MT; 50 μg/ml) for 24h to evaluate IDO-mediated T cell apoptosis. Apoptosis was assessed by AnnexinV and propidium iodine (PI) staining using the Annexin V-FITC apoptosis detection kit (BD Biosciences) as described by the manufacturer. Staining of the cells was evaluated by FACScan (Becton Dickinson). To investigate the effects of IDO on T cell proliferation 5 x 10^4 anti-CD3/anti-CD28 pre-activated CD4+ T cells were co-cultured with 1 x 10^4 DC in the presence or absence of 1-methyl-DL-tryptophan (MT; 50 μg/ml) for 3d and proliferation was assessed by the incorporation of [H]thymidine as described above.

Suppressor assay
On day 12, resting T cells were harvested and washed three times with serum-free medium. Cells (1x10^6) were stained with 1.0 μM CellTrace Far Red DDAO-SE (Molecular Probes Inc.,
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Eugene, OR), a fluorescent dye (FL4), for 15 minutes at room temperature according to the manufacturer’s instructions. After thorough washing, 2.5x10⁶ DDAO-SE labeled CD4⁺ T cells (DC–primed T cells) were stimulated by anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) in round-bottom 96-well plates. After overnight pre-activation 2.5x10⁵ peripheral CD4⁺ T cells were added, representing the responder T cells. Prior to this, the responder T cells were labeled with CFSE (0.5 μM; Molecular Probes), a green cell cycle tracking dye (FL1), for 15 min at room temperature. After 5 days, the content of DDAO-SE and CFSE in the DC-primed and responder T cells, respectively, was analyzed by flow cytometry, and the proliferation index or precursor frequency was determined with Modfit (BD Pharmingen) as described earlier (34).

Statistical analysis
Data were analyzed for statistical significance (GraphPad, InStat, version 2.02) using ANOVA or Student’s t test. A p value <0.05 was taken as the level of significance.

RESULTS
CD40 ligation on DC results in high levels of IDO
It has previously been shown that CD40 ligation, which activates both the canonical and the non-canonical NF-κB pathway, strongly increases IDO expression in human DC and macrophages (3,9,10,35), although there is some controversy on this issue (2,36). To confirm the ability of human DC to express IDO upon CD40 ligation, human monocyte-derived DC were stimulated either with CD40L or with LPS, which mainly activates the canonical NF-κB pathway (24). After 2 days, DC were lysed and IDO protein levels in the cytoplasmic extracts determined by Western blotting. In these experiments CD40L stimulation resulted in marked IDO expression in DC, in contrast to stimulation with LPS, which did not induce IDO (Figure 1A). LPS priming of DC, followed by CD40L stimulation, resulted in IDO expression comparable to CD40 ligation alone, showing that LPS does not irreversibly impair IDO induction by CD40L (data not shown). Furthermore, equally high IDO levels were detected in DC stimulated with soluble CD40L trimers, CD40L-expressing activated CD4⁺ T cells or CD40L-transfected J558 cells (Figure 1B), whereas mock-transfected J558-cells did not induce IDO. J558-CD40L cells were used for CD40 ligation in the remainder of the experiments. The CD40L-induced IDO protein was enzymatically active as shown by its ability to degrade the essential amino acid tryptophan into kynurenine (Figure 1C). IDO activity could be blocked by the addition of the competitive inhibitor 1-methyl-tryptophan (MT) (p<0.05). Next, we performed co-culture experiments to show that CD40L-stimulated IDO-expressing DC induced more apoptosis in activated T cells compared to LPS-stimulated DC (p<0.05), which could be abolished by addition of MT to the culture (Supplementary Figure 1A). Furthermore, blocking of IDO activity by MT in co-cultures of CD40L-stimulated DC and pre-activated CD4⁺ T cells resulted
in increased T cell proliferation (Supplementary Figure 1B). In addition, CD40L stimulation also increased IDO expression in human BDCA1+ myeloid DC and BDCA4+ plasmacytoid DC, freshly isolated from peripheral blood (Supplementary Figure 1C), underlining that CD40L induces IDO expression in naturally occurring DC as well. Altogether, these findings stress that CD40L induces high levels of IDO in human DC, which is enzymatically active and down-regulates effector T cell responses.

NBD peptide selectively blocks canonical CD40L-induced NF-κB activation

To verify that CD40L activates both the canonical and non-canonical pathways of NF-κB activation, while LPS only activates the canonical pathway in DC, we performed electrophoretic mobility shift assays (EMSAs) on the relevant NF-κB subunits. RelA is the transactivating subunit of the classical NF-κB heterodimer p50-RelA that is principally activated via the canonical pathway and is involved in many inflammatory processes, whereas RelB is the most promi-
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We observed comparable activation of RelA DNA-binding activity following CD40L and LPS stimulation, as indicated by an equal anti-RelA-induced supershift in the EMSA (Supplementary Figure 2). In contrast, RelB DNA binding was markedly induced by CD40L, but not by LPS stimulation.

The NBD peptide is a highly selective inhibitor of the canonical NF-κB pathway (29). We have previously demonstrated that the NBD peptide blocks LPS-induced activation of the canoni-
Table 1. Essential requirement of the non-canonical NF-κB pathway for regulatory mechanisms in DC, including induction of IDO

To first investigate the contribution of the canonical NF-κB pathway to CD40L-induced IDO expression, we tested the effects of NBD treatment on IDO protein levels in DC. We have pre-
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Figure 4. Essential requirement of the non-canonical NF-κB pathway for CD40L induced IDO in DC.

(A) Immature DC were treated with control non-blocking siRNA (siC) or siRNA for the non-canonical NF-κB pathway associated kinases NIK (siNIK) and IKKα (siIKKα). Subsequently, cells were matured for 2 days with CD40L, extensively washed and lysed in sample buffer. Cell lysates were analyzed by western blotting for IKKα and IDO content, and β-actin as loading control. Densitometry was performed on IDO blots; numbers indicate percent reduction of IDO expression by siIKKα. One representative experiment out of three is shown; densitometry includes data from all experiments (*p<0.01; **p<0.001). (B) Increased IL-12p70 production by DC after siRNA mediated knock-down of the non-canonical pathway. Immature DC were pre-incubated with NBD/MUT peptides and matured for 2 days with CD40L. After 48 h, the cells were thoroughly washed and stimulated with CD40L-expressing mouse plasmacytoma cells. Supernatants were harvested after 24 h and secreted IL-12p70 was measured by ELISA. Results are expressed as mean ± SD from one representative experiment of three performed in triplicate (*p<0.001). (C) Increased IL-6 production by DC after siRNA mediated knock-down of the non-canonical pathway. Immature DC were pre-incubated with NBD/MUT peptides and matured for 2 days with CD40L. After 48 h, the cells were thoroughly washed and stimulated with CD40L-expressing mouse plasmacytoma cells. Supernatants were harvested after 24 h and secreted IL-6 was measured by ELISA. Results are expressed as mean ± SD from one representative experiment of three performed in triplicate (*p<0.001). (D) IDO mediated inhibition of pre-activated T cell proliferation is non-canonical NF-κB pathway dependent. Monocyte-derived DC were stimulated with CD40L in the presence or absence of methyl-tryptophan (MT). After 48 h, cells were co-cultured with anti-CD3/anti-CD28 pre-activated CD4+ T cells in the presence or absence of MT for 3d. Subsequently, T cell proliferation was evaluated by [3H]thymidine incorporation. Data are presented as mean cpm ± SD of triplicate cultures. Results are representative of three independent experiments (*p<0.05).
viously demonstrated that the NBD peptide blocks LPS-induced functional maturation of DC and the production of pro-inflammatory cytokines (30). Canonical NF-κB blockade in CD40L-stimulated DC showed comparable reduction in the expression of HLA-DR and co-stimulatory molecules and the production of IL-12, which are all key to T cell immunity (Supplementary Figure 3A,B). In contrast, canonical NF-κB blockade in CD40L- or LPS-stimulated DC (CD40L-NBD DC and LPS-NBD DC, respectively) did not affect IDO protein levels, either positively or negatively (Figure 3A). In addition, the activity of CD40L-induced IDO was not changed (Figure 3B). Taken together, these findings rule out an important role for the canonical NF-κB pathway in the induction of IDO following CD40L stimulation in DC.

To investigate the contribution of the non-canonical NF-κB pathway to CD40L-induced IDO expression, we tested the requirement of different signaling intermediates of this pathway. Therefore, we used siRNA technology to specifically knock-down the non-canonical pathway-associated kinases NIK and IKKα, and studied the consequences for IDO expression at the protein level. This technique always yielded transfection efficiencies of >95% (Supplementary Figure 4A). Knock-down of both NIK and IKKα in CD40L-stimulated DC resulted in significantly reduced IDO expression compared to the control non-functional siRNA treated cells (0.44 ± 0.03 vs. 1; p<0.01 and 0.19 ± 0.01 vs. 1; p<0.001, respectively) (Figure 4A, lane 2 and 3), demonstrating that IDO expression in DC requires activation of the non-canonical NF-κB pathway. No effects were observed of siRNA mediated knock-down of the non-canonical NF-κB pathway in J558-mock stimulated DC (Supplementary Figure 4B).

Simultaneously, knock-down of IKKα or NIK resulted in strongly increased pro-inflammatory IL-12p70 (*p<0.001) and IL-6 (*p<0.001) production in DC, which could be completely blocked by inhibition of the canonical pathway by the NBD peptide (Figure 4B,C). IL-10 production in CD40L-stimulated DC was just above background level and was not affected by inhibition of canonical and/or non-canonical NF-κB signaling (data not shown). These findings reveal a more general role for non-canonical NF-κB signaling in the negative regulation of inflammation, via down-regulation of pro-inflammatory cytokine production in DC.

Subsequently, we tested the functional consequences of reduced IDO expression in CD40L-stimulated DC on the proliferative response of effector T cells. siRNA-mediated knock-down of the non-canonical pathway resulted in significantly increased effector T cell proliferation (p<0.05) (Figure 4D). Blockade of IDO activity through addition of MT to the culture also resulted in a significant increase in effector T cell proliferation (p<0.05). Addition of MT did not further increase the effector T cell proliferation that was observed after knock-down of IKKα or NIK in DC. These results prove that, while the canonical NF-κB pathway in DC mediates T cell immunity, the non-canonical NF-κB pathway plays a crucial role in the negative regulation of effector T cell responses via the induction of IDO in DC and down-regulation of pro-inflammatory cytokine production in DC.
Role of the canonical and non-canonical NF-κB pathways in the initiation and regulation of immunity

To elucidate the consequences of CD40L-induced canonical and non-canonical NF-κB activation in DC for the ability of these DC to initiate adaptive immunity, we next investigated to what extent NBD or siIKKα treatment of CD40L-stimulated DC affects the capacity of DC to activate naive T cells. First we examined the potential of these DC to induce proliferation in naive T cells in a mixed lymphocyte reaction (MLR). Blockade of the canonical pathway by NBD peptide pre-treatment significantly reduced naive T cell proliferation in this model (Figure 5A). This finding may be explained by the inhibitory effects of the NBD peptide on
the expression of HLA-DR and co-stimulatory molecules, as well as reduced levels of the T cell stimulatory cytokine IL-12p70 in NBD-treated DC (Supplementary Figure 3A,B). However, selective knock-down of IKKα in DC did not affect naive T cell proliferation positively or negatively, neither in NBD treated DC nor in control DC (Figure 5B). In addition, NBD-treated DC did not increase apoptosis of naive CD4+ T cells and no effect of IKKα knock-down in DC on naive T cell apoptosis was observed (Figure 5C). In summary, canonical NF-κB inhibition in CD40L-stimulated DC results in the generation of immunomodulatory DC that lead to hypoproliferative naive CD4 T cells. These experiments demonstrate an important role for the canonical NF-κB pathway in DC in the initiation of T cell responses, whereas the non-canonical NF-κB pathway, which regulates effector responses, neither initiates nor regulates this process.

Essential requirement of the non-canonical NF-κB pathway in DC for the development of regulatory T cells

Regulatory T cells (Treg) are closely linked to the induction of IDO in DC, for instance through ligation of CTLA-4 (12), but whether IDO-expressing DC may also give rise to Treg has not been reported, thus far (reviewed in (2)). Treg have many different faces, but a general feature is their low, intrinsic proliferative capacity (reviewed in (37,38)). The low proliferative capacity of the T cells induced by CD40L-NBD DC (CD40L-NBD T cells; Figure 5A), in combination with the massive IDO expression in the instructing DC, could therefore point to the development of regulatory T cells with a suppressive effect on bystander T cells. Therefore, CD40L-NBD T cells were tested for their possible regulatory activity in a suppressor assay using two cell cycle tracking dyes as described previously (34). CD40L-NBD T cells clearly exhibited suppressive function, indicated by a slower rate of target cell division and progression through less cell cycles following anti-CD3/anti-CD28 stimulation in the co-culture (filled histogram) (Figure 6A). CD4+ T cells derived from CD40L-MUT DC (CD40L-MUT T cells) did not suppress target cell proliferation as the vast majority of the cells progressed through all cell cycles (open histogram). Analysis of the percentage of proliferated cells/cycle revealed that the percentage of target cells that progressed through five and six rounds of division was significantly reduced in the presence of CD40L-NBD T cells compared to CD40L-MUT T cells (22.39 ± 2.20 % and 4.61 ± 0.45 % for CD40L-NBD T cells, compared to 33.38 ± 0.60 % and 41.79 ± 1.97 % for CD40L-MUT T cells, for 5 and 6 rounds of division respectively (p<0.001) (Figure 6B). Setting the mean fluorescence intensity of the target cells cultured with T cells instructed by CD40L-stimulated DC without inhibitors at 100%, we calculated the percentage of target cell proliferation in the presence of either CD40L-NBD T cells or CD40L-MUT T cells. Addition of CD40L-NBD T cells to the target cells resulted in a dramatic reduction in target cell proliferation compared to incubation with CD40L-MUT T cells (46.1 ± 2.5 % vs. 109.1 ± 10.2 % respectively; p<0.001) (Figure 6C). These data demonstrate that CD40 ligation of DC in combination with canonical NF-κB inhibition results in the generation of regulatory T cells.
Induction of IDO in dendritic cells requires non-canonical NF-κB signaling

Figure 6. CD40L-NBD T cells suppress proliferation of CFSE labeled CD4+ target cells and are generated via a non-canonical NF-κB mediated mechanism in DC.

(A) CD40L-NBD T cells suppress proliferation of CFSE labeled CD4+ target T cells. Naive CD4+ T cells instructed by NBD treated CD40L stimulated DC (CD40L-NBD T cells) were tested for their suppressive activity on proliferation of anti-CD3/CD28 stimulated CFSE-labeled CD4+ target T cells. CFSE-profiles of target cells co-cultured with CD40L-NBD T cells (filled histogram), target cells co-cultured with CD40L-MUT T cells (black line) or target cells alone (grey line) are shown. Histograms are representative profiles from one out of five independent experiments that yielded similar results. (B) Quantification of percentage of proliferated target cells per cycle in cocultures with CD40L-NBD T cells or CD40L-MUT T cells. Data are expressed as percentage of proliferated target cells per cycle and represent mean ± SEM from five independent experiments. (C) Quantification of target cell proliferation in coculture with CD40L-NBD T cells or CD40L-MUT T cells. Data are expressed as % proliferation compared to control (medium+CD40L) and represent mean ± SEM from five independent experiments (*p<0.001). (D) Suppressive capacity of CD40L-NBD T cells is induced by DC via a non-canonical NF-κB, IDO mediated mechanism. CD40L-NBD DC were treated with siRNA for IKKα (siIKKα) or control siRNA (siC), subsequently the suppressive activity of T cells instructed by these DC (siIKKα or siC CD40L-NBD T cells respectively) was tested in the same assay as described above. CFSE-profiles of target cells co-cultured with siC CD40L-NBD T cells (filled histogram) and target cells co-cultured with siIKKα CD40L-NBD T cells (grey line) were compared with siC CD40L-MUT T cells (black line) and siIKKα CD40L-MUT T cells (stipled line). Shown are representative profiles from one out of three independent experiments that yielded similar results. (E) Quantification of percentage of proliferated target cells per cycle in cocultures with T cells derived from siRNA treated CD40L stimulated NBD/MUT DC. Data are expressed as percentage of proliferated target cells per cycle and represent mean ± SEM from three independent experiments. (F) Quantification of target cell proliferation in coculture with T cells derived from siRNA treated CD40L stimulated NBD/MUT DC. Data are expressed as % proliferation compared to control (medium+CD40L) and represent mean ± SEM from three independent experiments (*p<0.001).
from naive CD4+ precursors. No suppressor activity was observed in CD4+ T cells derived from naive precursors that interacted with LPS-NBD DC or LPS-MUT DC (data not shown), suggesting that non-canonical NF-κB signaling in CD40L-NBD DC is vital to the development of Treg. Indeed, IKKα knock-down in CD40L-NBD DC almost completely abolished the suppressive capacity of CD40L-NBD T cells, indicated by normal proliferation of target cells in the suppressor assay (Figure 6D; grey line) and the increase of the percentage of target cells that progressed through five and six rounds of division (from 18.82 ± 0.16 % to 34.78 ± 1.09 % and from 5.77 ± 0.37 % to 25.28 ± 1.68 %, respectively (p<0.001), compared to 34.58 ± 0.42 % and 37.32 ± 2.65 % in the MUT + siIKKα samples (Figure 6E). Target cell proliferation increased from 41.2 ± 3.7 % to 86.0 ± 2.5% after siIKKα treatment of DC (*p<0.001) (Figure 6F). siRNA treatment of DC by itself did not result in altered T cell proliferation of naive CD4+ T cells that could account for the observed differences (Supplementary Figure 5). Collectively, these data demonstrate an essential requirement of the non-canonical NF-κB pathway in DC for the generation of regulatory T cells from naive T cells.

DISCUSSION

In the current study, we demonstrate that the non-canonical NF-κB pathway is essential for IDO expression, and controls the production of pro-inflammatory cytokines induced by the canonical NF-κB pathway in CD40L-activated DC. We propose that the non-canonical NF-κB pathway, induced by CD40L expressed on T cells, controls T cell activation in vivo. The regulation of adaptive immunity by the non-canonical NF-κB pathway is substantiated by the finding that CD40L-induced IDO is enzymatically active, mediates apoptosis of effector T cells and, in combination with inhibition of the canonical pathway, promotes the development of regulatory T cells (Treg) from naive CD4+ T cell precursors. Activation of canonical NF-κB signaling is well-controlled by the induction of IkBα transcription following NF-κB DNA binding, which attenuates the activation of this important pro-inflammatory pathway. To date, immune regulation by products of the non-canonical NF-κB pathway is largely unknown, except for a recent study in macrophages that suggests a role for IKKα in the negative regulation of inflammation via accelerating the turnover of pro-inflammatory RelA and c-Rel-containing dimers and their removal from pro-inflammatory gene promoters (26). We found that selective knock-down of the non-canonical pathway using siRNA for IKKα or NIK in DC also resulted in increased pro-inflammatory cytokine production, suggesting a similar negative regulation also takes place in DC. Our present data also indicate that IKKα mediated IDO expression could provide an additional mechanism of immune regulation, as IDO does not only prevent further activation of DC via tryptophan catabolism (11), but also regulates adaptive immunity induced by these DC by controlling the size and the activity of the induced effector T cell population. Based on these findings we propose that non-canonical NF-κB signaling in
DC is important in the negative regulation of inflammation and immunity by IDO-expressing cells.

CD40L-stimulation in combination with specific inhibition of canonical, IKK complex-mediated NF-κB activation resulted in the generation of a regulatory DC phenotype. These cells were characterized by low expression of MHC class II, costimulatory molecules like CD86 and cytokines, such as IL-12p70, but unaltered expression of IDO. These DC had a poor capacity to induce proliferation in naïve T cells, which was not caused by IDO expression (data not shown) and may very well be the result from poor MHC class II and CD86 expression (39), but promoted the development of Treg in these T cells, which was entirely dependent on non-canonical NF-κB signaling. Based on these findings we propose that non-canonical NF-κB signaling in DC negative regulates immunity through the induction of Tregs.

Various Treg categories have been described, like naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T cells or extrathymically induced Tr1 and Th3 cells (reviewed in (37,38)). Whereas induced Treg mainly exert their function via the local secretion of cytokines or cellular factors like CTLA-4, the suppressive mechanism of CD4<sup>+</sup>CD25<sup>+</sup> T cells remains elusive (37). The CD40L-NBD Treg do not seem to have clear characteristics of known Treg categories. They hardly produce IL-10, and addition of neutralizing antibodies against IL-10 and TGF-β did not inhibit the suppressive function of the Treg (data not shown). Furthermore, CD40L-NBD Treg did not differentially express the Treg marker FoxP3 or the surface markers GITR, CD103 and Lag3. Also, CD25 and CTLA-4 expression could not be linked conclusively with suppressive activity of the Treg (data not shown). Because CD40L-NBD Treg do not express higher levels of CD25 and proliferate less rapidly than control T cells, it is unlikely that these Treg exert their suppressive activity via consuming IL-2 in the culture leading to reduced T cell proliferation of the CD4<sup>+</sup> target cells in the suppressor assay. Interestingly, CD40L-NBD Treg produce less IFN-γ and more IL-4 (Supplementary Figure 6), a cytokine that has been associated with maintenance and even enhancement of regulatory T cell function (40-43). Furthermore, induced Treg have often reported to be IL-4 positive (44-48). Since the increased frequency of IL-4 producing T cells was not observed in co-cultures of naïve T cells with LPS-NBD DC, which like CD40L-NBD DC also exhibited reduced expression of MHC class II, costimulatory molecules and IL-12, it is probable that an IDO-related mechanism accounts for this increased IL-4 production. This finding is in line with earlier observations that IDO is able to induce apoptosis selectively in Th1 cells, but not in Th2 cells (6). In short, the NBD-CD40L DC derived-Treg are induced via a non-canonical NF-κB dependent, IDO-mediated mechanism, but the exact phenotype of the Treg and their mechanism of suppression requires further investigations. Nevertheless, these induced Treg promise to have great potential for cellular immunotherapy using DC.
In the current study we demonstrate that the regulatory properties of CD40L-stimulated IDO-expressing DC are optimal when canonical NF-κB activity is suppressed. We propose that the balance between the expression of inflammatory and T cell stimulatory molecules on the one hand, and tolerogenic molecules like IDO on the other hand, is of key importance for the capacity of DC to induce or suppress immunity. All known activators of the non-canonical NF-κB pathway simultaneously activate the canonical pathway (reviewed in (24)), but whether these factors also induce IDO remains to be investigated. However, the IKKα-mediated accelerated turnover of pro-inflammatory canonical NF-κB dimers will certainly add to the control of immune responses. We propose that selective inhibition of canonical NF-κB signaling will tip the balance and results in a relative increase in mechanisms associated with non-canonical NF-κB signaling, such as IDO expression after CD40 ligation. Another possible explanation for the enhanced effects of IDO in NBD-treated DC may be the fact that IL-6 has been demonstrated to negatively regulate IDO expression through the induction of SOCS3, thereby preventing tryptophan catabolism (49). Canonical NF-κB blockade results in reduced IL-6 production in DC, which will therefore result in increased IDO activity. Therefore, antigen presented by IKKβ-inhibited DC under inflammatory conditions in the abundant presence of CD40L-expressing T cells, may lead to tolerance induction and the development of Treg. This can be exploited in a number of therapeutic settings, for example in treatment of autoimmune diseases like rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis, as well as in allergy or transplant rejection. Specific inhibition of the canonical pathway-associated kinase IKKβ, while leaving the non-canonical pathway intact, would tip the balance towards regulatory rather than effector mechanisms. Indeed, IKKβ inhibition in DC has been demonstrated to result in immunoregulation both in vitro (42,50) and in vivo (51). Here, we provide evidence that the induction of such Treg occurs via non-canonical NF-κB signaling in DC. Conversely, when swift immune activation is required, for example in severe infections, IKKα inhibitors may be applied to boost the innate immune response via both IDO inhibition and the recently discovered prevention of RelA and c-Rel turnover, resulting in protracted NF-κB activation (26).

In conclusion, we demonstrate an essential requirement of the non-canonical NF-κB pathway for effective IDO induction in DC following CD40 ligation and an important role in the negative regulation of pro-inflammatory cytokine production. Selective canonical NF-κB inhibition results in DC that induce regulatory T cells via a non-canonical pathway-dependent IDO-mediated mechanism. This discovery presents a novel mechanism of action via which IKKβ inhibitors may exert their beneficial effects and has important implications for the use of anti-inflammatory drugs which selectively inhibit the canonical NF-κB pathway and therefore would be beneficial for immunotherapy of transplantation, autoimmune and allergic diseases.
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SUPPLEMENTARY FIGURES

Supplementary Figure 1. CD40L-induced IDO in DC down-regulates effector T cell responses and CD40L stimulation of BDCA1+ and BDCA4+ DC also results in increased IDO expression.

(A) CD40L-induced IDO induces apoptosis of pre-activated T cells. Monocyte-derived DC were stimulated with CD40L or LPS in the presence or absence of MT. After 48 h, cells were co-cultured with anti-CD3/anti-CD28 pre-activated CD4+ T cells in the presence or absence of MT for 24 h. Subsequently, T cell apoptosis was assessed by Annexin V/propidium iodine (PI) staining and evaluated by flow cytometry. Results represent mean ± SEM from 3 independent experiments (*p < 0.05). (B) CD40L-induced IDO reduces proliferation of pre-activated T cells. Monocyte-derived DC were stimulated with CD40L or LPS in the presence or absence of MT. After 48 h, cells were co-cultured with anti-CD3/anti-CD28 pre-activated CD4+ T cells in the presence or absence of MT for 3d. Subsequently, T cell proliferation was evaluated by [3H]Tdr incorporation. Data are presented as mean cpm ± SD of triplicate cultures. Results are representative of three independent experiments (*p < 0.05). (C) CD40L stimulation of BDCA1+ and BDCA4+ cells also results in IDO protein expression. BDCA1+ and BDCA4+ DC were freshly isolated from peripheral blood and stimulated with CD40L expressing J558 cells. After 48 h, the cells were extensively washed and lysed in sample buffer. Cell lysates were analyzed by Western blotting for IDO content. Representative blots from four independent experiments are shown.
Supplementary Figure 2. CD40L activates both canonical and non-canonical NF-κB in DC.

(A) NF-κB electrophoretic mobility shift assay (EMSA). Monocyte-derived DC were either left unstimulated or stimulated with LPS or CD40L for 4h. NF-κB EMSA was performed on nuclear extracts of unstimulated, LPS- or CD40L-stimulated DC. Anti-RelA and anti-RelB supershift antibodies were added 5 min after the start of the binding reaction. NF-κB-binding activity was analyzed using the following double-stranded NF-κB consensus binding-site oligonucleotides (BioSource) (consensus sequence is underlined): 5’-AGTTGAGGGGACTTTCCCAGGC-3’. Results shown are from 1 experiment, representative for 3 independent experiments. (B) SP-1 EMSA. Monocyte-derived DC were either left unstimulated or stimulated with LPS or CD40L for 4h. EMSA for the constitutive transcription factor SP-1 was performed on the same nuclear extracts of unstimulated, LPS- or CD40L-stimulated DC used for the NF-κB EMSA. Equal protein loading was checked by testing for binding of the constitutive transcription factor SP-1 using the herpes simplex virus SP-1 site containing oligonucleotide 5’-CCGGCCCCATCCCCGGCCGCCCCCCATCC-3’. Results shown are from 1 experiment, representative for 3 independent experiments.
Supplementary Figure 3. Selective blockade of the canonical NF-κB pathway inhibits DC maturation.

(A) NBD peptide blocks both LPS- and CD40L-induced up-regulation of MHC class II and co-stimulatory molecules. Immature DC were cultured with LPS or CD40L in the presence or absence of NBD/MUT peptides. After 2 days, DC were analyzed for the expression of cell surface molecules HLA-DR, CD83, and CD86 by flow cytometry. The filled histograms represent the specific expression of surface markers on DC incubated with NBD and the open histograms represent control DC incubated with MUT peptides. NBD peptide treatment abrogated the up-regulation of HLA-DR (p<0.05), CD83 (p<0.05), and CD86 (p<0.05) after LPS (upper panels) and CD40L stimulation (lower panels). In contrast, the MUT peptide did not influence CD40L-induced up-regulation of HLA-DR, CD83, and CD86. These data show that inhibition of the canonical NF-κB pathway by the NBD peptide alters the maturation state and co-stimulatory molecule expression of LPS and CD40L stimulated DC to the same extent. Data are representative of at least five independent experiments.

(B) Pro-inflammatory cytokine production by DC is dose-dependently inhibited by the NBD peptide. Immature DC were pre-incubated with the indicated concentrations of NBD/MUT peptides and matured for 2 days with LPS or CD40L. After 48 h, the cells were thoroughly washed and stimulated with CD40L-expressing mouse plasmacytoma cells. Supernatants were harvested after 24 h and secreted IL-12p70 was measured by ELISA. Results are expressed as mean ± SD from one representative experiment of three performed in triplicate.
Supplementary Figure 4. Transfection efficiency of siRNA in DC.

(A) To evaluate transfection efficiency, immature DC were incubated with FAM-labeled control RNA siRNA in Lipofectamine 2000 for 4 h at 37°C. After that, serum concentration was adjusted to 10% by addition of IMDM containing 50% FCS. After 24 h transfected DC were analyzed by flow cytometry for FAM content. Histogram shows fluorescence in the FL1-channel of untransfected DC (black line), control siRNA transfected DC (grey line) and FAM-labeled RNA transfected DC (filled histogram). We routinely obtained transfection efficiencies of 90-95%. A representative example out of more than five independent experiments is shown. (B) No effect of siRNA mediated knock-down of the non-canonical NF-κB pathway in J558-CD40L or J558-mock cells, extensively washed and lysed in sample buffer. Cell lysates were analyzed by western blotting for IDO content. One representative experiment out of three is shown.

Supplementary Figure 5. siRNA treatment does not change the capacity of DC to induce T cell proliferation.

Immature DC were either untreated or treated with siRNA, incubated with NBD/MUT peptides and matured for 2 days with CD40L. Subsequently, the cells were thoroughly washed, loaded with SEB and used to stimulate naive CD4+ T cells. The proliferative response was determined at day 5 of coculture by [3H]Tdr incorporation. Data are presented as mean cpm ± SD of triplicate cultures. Results are representative of three independent experiments.
Supplementary Figure 6. Blockade of the canonical NF-κB pathway in DC alters T cell polarization.

Naive T cells that encounter CD40L-NBD DC develop into Th2 cells. Naive CD4+ T cells were stimulated with SEB-loaded DC that were earlier stimulated with LPS or CD40L in the absence or presence of NBD peptides. T cells from the MLR were expanded at day 5, using IL-2 and IL-15, followed by re-stimulation of the proliferated T cells after 12 days with PMA/ionomycin. Single-cell IL-4 and IFN-γ production was measured by intracellular flow cytometric analysis to determine the percentage of IL-4- and IFN-γ-producing T cells after DC instruction. Clearly, NBD treatment of DC affected the cytokine balance of the effector T cells. Like for LPS-matured DC, NBD treatment of CD40L-matured DC resulted in a strongly reduced percentage of IFN-γ-producing T cells. However, in contrast to LPS-NBD DC derived T cells (LPS-NBD T cells), CD40L-NBD DC derived T cells (CD40L-NBD T cells) revealed a significant shift in the Th1/Th2 profile towards a more Th2-like phenotype demonstrated by an increase in IL-4 expression. Calculated over all experiments, the percentage of IFN-γ+ T cells dropped with 62.5 ± 18.8%, whereas the percentage of IL-4+ T cells increased dramatically (p<0.001). When MUT-treated DC were used to stimulate naive T cells, no difference was observed in the development of IL-4- or IFN-γ-producing T cells compared to stimulation with untreated DC. These data demonstrate that CD40L-NBD DC block Th1 polarization and favor Th2 differentiation. This suggests that canonical NF-κB inhibition by the NBD peptide, in combination with CD40L-induced non-canonical pathway-associated mechanisms, such as the induction of IDO, results in increased Th2 instruction by DC. Shown are flow cytometry plots from one representative experiment out of at least five, performed in duplicates.
Chapter 11

General discussion and summary
This thesis focuses on the feasibility of NF-κB inhibition as a therapy for rheumatoid arthritis (RA) and validates IKKβ as a therapeutic target, using both gene therapy and small molecule inhibitors. In addition, the expression of dendritic cells (DC) in RA synovial tissue (ST) and the consequences of NF-κB inhibition in DC, as an initial step towards the development of cell-based immunotherapy for RA, are investigated.

BACKGROUND

RA is a chronic autoimmune disease that affects approximately 1% of the population (1). Key features of the disease include painful, swollen joints and disability. Although the etiology of RA is still largely unknown, treatment of this disease has been improved over the years due to important advances in the field. RA treatment consists of non-steroidal anti-inflammatory drugs (NSAIDs) together with disease-modifying antirheumatic drugs (DMARD’s), like methotrexate. In patients who do not respond adequately, this treatment is increasingly combined with so-called “biologicals” that include antibodies or soluble receptors that specifically target a certain pro-inflammatory protein like TNFα. These therapies are very effective in reducing arthritis severity and often prevent the irreversible joint destruction (reviewed in (2)). Nevertheless, a substantial percentage of RA patients do not respond to these new treatments for RA. Therefore, it is necessary to develop new treatment options for RA. Ideally, new therapies should specifically block the inflammatory response in the joint, while normal immune responses to pathogens remain intact and side-effects are limited. Specific targeting of intracellular signal transduction intermediates or transcription factors involved in the initiation and perpetuation of synovial inflammation appears to be an elegant and powerful way to combat the pathological cellular processes observed in arthritis.

In RA synovium the NF-κB family of transcription factors is highly activated and can induce transcription of pro-inflammatory cytokines, adhesion molecules and inducible nitric oxide (3,4), thereby contributing significantly to the RA disease process. IKKβ, the main NF-κB activating kinase in response to pro-inflammatory cytokines, is also highly expressed in human RA ST and correlates significantly with the expression of phosphorylated (ph) IκBα (J. Ludikhuize et al. Manuscript in preparation). Furthermore, high expression of IKKβ and phIκBα correlates with erosive disease and radiological progression of joint destruction after 1 year. These findings support the view that IKKβ plays a central role in the pathogenesis of arthritis. We propose that canonical NF-κB activation contributes to synovial inflammation and that selective targeting of IKKβ may inhibit synovial inflammation and could perhaps protect against joint destruction.
In **Chapter 1**, an introduction is provided about the present knowledge of the etiology and pathogenesis of RA. Furthermore, the two NF-κB signal transduction pathways are introduced, focusing on the role of this important transcription factor family in RA. Moreover, strategies to inhibit NF-κB locally in the joint are presented, emphasizing the use of small molecule compounds and gene therapy. Finally, the role of DC in RA and the available options to generate tolerogenic DC to modulate immune responses are discussed. **Chapter 2** reviews the current literature on major signal transduction pathways involved in the pathogenesis of RA and the recent advances in targeting a number of key intracellular pathways, most notably the NF-κB pathway.

**MAIN FINDINGS**

We have set up immunohistochemical staining procedures to detect cytokines and matrix-metalloproteinases on paraffin embedded joints from rats with adjuvant arthritis (AA). These rats were treated with an adenoviral vector containing the dominant negative IKKβ gene (Ad.IKKβdn), which ameliorated arthritis (5) and resulted in reduced synovial inflammation. Digital image analysis of the immunohistochemical stainings not only allowed for quantification of specifically stained markers, but could also be used to determine synovial cellularity. This detection method is reported in **chapter 3** and was used throughout the other chapters of this thesis.

Viral-mediated gene transfer is currently the most efficient system for delivering therapeutic proteins *in vivo* and rAAV is considered the most promising viral vector for gene therapy in RA (6). To date, most gene therapy studies with recombinant (r)AAV were carried out using serotype 2, but recent studies using vectors derived from alternative rAAV serotypes have shown improved potency and altered tropism of the rAAV vector by packaging the same vector genome with different AAV capsids, signifying that the transduction efficiency of AAV serotypes is highly tissue and cell type specific (reviewed in (7)). In **chapter 4** we therefore tested five different AAV serotypes (AAV1-AAV5) for their efficiency to transduce ST, the key target for gene therapy in RA, in AA. The AA model was used because it is one of the best validated animal models for RA and the size of the intra-articular space of the ankle joint allows reliable intra-articular injection (8,9). Our data clearly demonstrate that AAV5 shows the highest transduction efficiency in rat synovium compared to the other serotypes tested, both on the mRNA level and the protein level. In addition, we showed that AAV5 also exhibits high transduction efficiency of human RA fibroblast-like synoviocytes (FLS). This had major implications for our research as we chose to use AAV5 as vector in our studies, instead of the so far widely used vector AAV2.
In chapter 5, the effect of local gene therapy using rAAV5 containing the dominant negative IKKβ gene (AAV5.IKKβdn) was tested in AA. In the rats treated with AAV5.IKKβdn in early arthritis significantly reduced paw swelling was observed. Immunohistochemical analysis of ST revealed strongly reduced levels of pro-inflammatory cytokines, whereas IL-10 levels were not affected. In addition to the animal studies, it was demonstrated for the first time that AAV5 is capable of transducing human whole ST biopsies ex vivo, resulting in reduced pro-inflammatory cytokine production. In conclusion, rAAV5 can be used to successfully target an intracellular signaling intermediate like IKKβ in the synovium, resulting in reduced severity of inflammation in AA in vivo and pro-inflammatory cytokine production in human RA ST ex vivo. This translational research represents a crucial next step in the development of gene therapy for (future) application in humans. The feasibility and safety of AAV mediated gene therapy in humans has been investigated mainly for the (transient) correction of disorders like cystic fibrosis (10) and hemophilia B (11). A clinical trial of intra-articular gene therapy for RA using AAV2 encoding a potent inhibitor of TNFα known as TNFR:Fc is currently ongoing (12), and thus far no adverse effects have been reported. Although these and other recent advances in the field have made gene therapy a very promising new treatment option for RA, more pre-clinical research needs to be done before this new approach may be of benefit for RA patients.

In addition to gene therapeutic approaches to inhibit NF-κB, we also investigated the use of low-molecular compounds in RA. Compared to gene therapy these compounds require repetitive administration, but while AAV gene therapy results in differential transduction of cell types due to differences in surface expression of co-receptors that are required for AAV cell entry (7), low-molecular compounds have the advantage to target NF-κB in almost all cells since they usually contain a membrane translocation sequence for cell entry. Therefore, we performed in vitro and in vivo experiments with several new small molecule NF-κB inhibitors. At present, the NBD peptide is one of the best characterized specific IKKβ inhibitors, both in vitro and in vivo (13,14). We used this highly selective IKKβ inhibitor to investigate whether direct intra-articular injection of small molecule NF-κB inhibitors can modulate the immune response in arthritis. In chapter 6 we describe the beneficial effects of local NF-κB inhibition in AA, using the specific IKKβ blocking NBD peptide. Intra-articular injection of the NBD peptide at the onset of disease led to significantly reduced severity of arthritis and less bone destruction. Bone destruction is usually evaluated on plain X-rays of the ankle joints using a validated scoring system as described previously (15). However, the improvement of imaging techniques continues and computed tomography (CT) generates high resolution images of the joints that permit even better evaluation of bone destruction, because ectopic bone formation and small erosions can be detected more accurately (Figure 1). Although this technique is still relatively time consuming and scoring systems need further validation, we expect that future studies focusing on the effects of new experimental therapies on bone
destruction will increasingly use CT analysis or other high resolution imaging systems. In addition to the beneficial effects on arthritis severity and bone destruction, intra-articular injection of the NBD peptide was associated with decreased synovial cellularity and reduced expression of pro-inflammatory cytokines in the synovium, as determined by the methods described in chapter 3. The NBD peptide also blocked cytokine production in human macrophages and RA FLS in vitro. Importantly, the NBD peptide significantly decreased cytokine production by human RA whole ST biopsies. Taken together, these results indicate that, in addition to the gene therapeutic approach, IKKβ targeted NF-κB blockade using the small molecule NBD peptide could offer a new opportunity for the local treatment of arthritis.

At present, there is a lot of interest in cell-based therapies for inflammatory disorders, especially in the ex vivo manipulation of dendritic cells (DC) to induce tolerance leading to remission or immunoregulation of autoimmune diseases (16-19). Because of this, we extended our research to this field. Naturally occurring DC can be divided into different subsets, of which myeloid DC (mDC) and plasmacytoid DC (pDC) are two major categories. In the study described in chapter 7 it was found that the number of pDC was significantly higher than the number of mDC in RA ST, and that these pDC showed significantly higher expression of the maturation marker CD83. Furthermore, in RA ST both mDC and pDC co-localised with T cell aggregates containing both CD3+ and CD8+ cells. These findings indicate that the maturation status and localization of mDC and pDC in ST may play an important role in synovial inflammation possibly via stimulation of memory T cells or the induction of (clonal) expansion in naive T cells. Moreover, the release of pro-inflammatory cytokines by both DC subsets may
sustain the inflammatory process observed in patients with RA. Conceivably, immunomodulation by specifically targeting ST DC or ex vivo modulation of DC followed by reinfusion may provide a novel antirheumatic strategy.

NF-κB inhibition is one of the most powerful ways to generate tolerogenic DC that could be exploited to treat a variety of autoimmune diseases, like RA, or to interfere with allograft rejection after transplantation (20). Based on these findings, the effects of NF-κB blockade in monocyte-derived DC as a model system to study DC biology were investigated, as an initial step towards the development of cell-based immunotherapy for RA (Figure 2). In chapter 8 the effects of caffeic acid phenethyl ester (CAPE) on DC were explored. CAPE exerts its anti-inflammatory effects in part by anti-oxidant activities (21), but it also acts as a potent inhibitor of NF-κB activation by preventing IkBα degradation (22) and blocking of p65 nuclear translocation and DNA binding (23). We found that CAPE treatment of DC resulted in an immature phenotype with decreased production of the T cell instructing cytokine IL-12. Furthermore,
interaction of CAPE-treated DC with naive T cells caused less formation of effector T cells. Surprisingly, CAPE treatment concomitantly increased functional chemokine receptor (CCR7) expression on DC, resulting in enhanced migration of these cells to the CCR7 ligands CCL19 and CCL21. This could have important implications for the use of these cells in cell-based immunotherapy. The enhanced CCR7 expression would likely result in increased migration of these immature DC to inflammatory sites, thereby predominantly dampening the immune response at the site of inflammation.

In chapter 9 it is shown that more specific blocking of NF-κB in DC using the NBD peptide results in a less mature phenotype leading to decreased inflammation and even tolerance by a reduction in cytokine production and effector T cell formation. However, CCR7 expression was not significantly increased (data not shown). Interestingly, in chapter 10 we extended these findings and found that inhibition of the canonical NF-κB pathway using the NBD peptide, in combination with triggering of the non-canonical NF-κB pathway by CD40L in DC results in the expression of the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO), not only in monocyte-derived DC but also in naturally occurring mDC and pDC. This results in the generation of potent regulatory T cells (Treg) that suppress proliferation of effector T cells. Consequently, this has important implications for the use of anti-inflammatory drugs that, like the NBD peptide, selectively inhibit the canonical NF-κB pathway and would therefore be beneficial in immunotherapy of transplantation, autoimmune, and allergic diseases. Recently, it has been described that cellular immunotherapy using DC in which the canonical NF-κB pathway was selectively blocked by dominant-negative IKKβ gene therapy resulted in the formation of potent CD4+ Treg that prevented transplant rejection in vivo (24), indicating this approach could be feasible in autoimmune diseases like RA as well.

The experimental approaches to generate DC for immunotherapy described above are based on in vitro generation of tolerogenic DC via pharmacological or gene therapy-mediated NF-κB inhibition, followed by intravenous administration of the DC. This technique has several possible disadvantages for application in the clinic: (I) generation of DC from monocytes requires a minimum of 5-7 days culture in vitro which may lead to genetic alterations in the cells; (II) tolerogenic immature DC are unstable and easily mature in vitro and in vivo, which would have adverse effects in autoimmune diseases; (III) only a very small fraction of the injected DC traffic to secondary lymphoid organs and interact with naïve T cells (reviewed in (25)). To avoid these potential drawbacks, it would be extremely valuable to develop techniques to generate tolerogenic DC via in situ targeting of DC in vivo. This could be achieved by specific targeting of NF-κB inhibitors to DC in vivo. Unfortunately, this is not easy. Intravenous injection of antibodies that recognize specific markers on the DC surface coupled to antigens have been used as a strategy to specifically target DC in vivo (26-28). Although this technique is suitable for directing antigens to DC, therapeutic proteins targeted to DC in this way end
up in the lysosomal compartment rather than free in the cytoplasm. *In vivo* targeting of the NF-κB inhibitor NBD peptide to DC by this technique would for example lead to lysosomal processing and degradation of the peptides thereby disabling their function. Therefore, extra efforts are needed to discover the Holy Grail that allows specific targeting of immunmodulatory proteins to DC *in vivo*.

**CONCLUSION AND FUTURE PERSPECTIVES**

We demonstrated that the pro-inflammatory transcription factor NF-κB, and in particular the important activating kinase IKKβ, is a good therapeutic target in RA, using both a gene therapy approach and small molecule inhibitors. Direct intra-articular injection of the vector or the small molecule inhibitors into the ankle joints of arthritic rats significantly ameliorated established arthritis. Furthermore, we have shown that these strategies could also be used to inhibit inflammatory responses in human RA synovial cells cultured *in vitro*, as well as whole synovial tissue biopsies *ex vivo*. Collectively, these studies indicate that IKKβ could be a good therapeutic target in RA patients as well. At present clinical trials using AAV2 gene therapeutic approaches are carried out in RA patients, and so far seem to be safe and well tolerated (12). We envision that local gene therapy for RA patients could be improved using AAV5 and that this offers a valuable new approach for the local treatment of arthritis. Clearly, there is a need for optimizing vectors for RA gene therapy in order to achieve highly efficient transduction of the ST, preferably in long-lived FLS, to ensure stable expression of the therapeutic transgene over time. Furthermore, regulation of transgene expression is required to avoid possible toxicity. Preferably, expression of the therapeutic protein is self-regulated, for example by using an NF-κB responsive promoter. This promoter induces expression of the therapeutic protein under inflammatory conditions, while expression is shut-off when inflammation is absent. Thus, an auto-regulatory process is achieved with elegant control of inflammation. In addition to optimizing the vector, new potential therapeutic targets arise continuously. Therefore, the anti-arthritic capacity of new constructs should be determined *in vivo*. In this respect it can be envisioned that constructs encoding secreted therapeutic proteins, such as soluble TNFα blockers, may be more effective in the long-term suppression of inflammation than constructs that target intracellular proteins, (I) because the therapeutic protein exerts its effects also on cells other than the transduced cell alone, and (II) targeting of intracellular molecules, like signal transduction mediators of the NF-κB pathway, may result in apoptosis of the transduced cell, thereby preventing stable longterm expression of the therapeutic transgene.

Small molecule NF-κB inhibition using the NBD peptide was shown to be effective in reducing inflammation in animal models of arthritis and human synovial biopsies. We tested one of the
best small molecule IKKβ inhibitors for intra-articular use in arthritis, but these compounds are also being improved to enhance bio-availability and specificity, in order to limit possible side-effects. Therefore, future research will determine the optimal approach to inhibit IKKβ. In conclusion, our work has opened the path towards a more elegant intra-articular treatment of arthritis, with perhaps less side-effects.

In addition to the central role of IKKβ in arthritis, we established an important role for the alternative NF-κB pathway in DC in the negative regulation of inflammation through the generation of regulatory T cells (Figure 3). However, the mechanism used by these regulatory T cells to suppress proliferation of effector T cells is not fully understood. Therefore, additional

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**Figure 3. Model of T cell instruction by dendritic cells (DC).**

Under inflammatory conditions mature DC generate effector T cells. The induction of these cells from naive precursors is dependent on IKKβ in DC (described in Chapter 9 of this thesis). By contrast, DC presenting antigen in a setting without pro-inflammatory cytokines and co-stimulatory molecules, or perhaps without effective presentation of peptide–MHC, induce regulatory T cells (Treg) that suppress immune responses. This mechanism is mediated by non-canonical NF-κB signaling that gives rise to IDO in CD40L stimulated DC (described in Chapter 10 of this thesis).

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**Figure 4. Schematic representation of the role of IKKα and IKKβ in inflammation**

(A) In steady state conditions canonical NF-κB signaling (IKKβ) is balanced by non-canonical NF-κB signaling (IKKα). (B) Inflammation results in IKKβ-mediated processes that cannot be balanced by IKKα activation. When IKKβ activity is inhibited (C), the balance shifts towards IKKα-mediated processes, including the induction of IDO or negative regulation of pro-inflammatory cytokine production, that ultimately lead to resolution of inflammation.
work is required to elucidate the mechanism of T cell suppression that is induced by NBD-treated regulatory DC in T cells, before these immunoregulatory DC could be used safely for cell-based immunotherapy of autoimmune diseases. In Figure 4 we propose a unifying mechanism for the role of the two NF-κB activation pathways: the canonical pathway that is activated via IKKβ and the non-canonical pathway that is dependent on IKKα. The pro-inflammatory role of IKKβ has been well established (5,29-32). Based on the findings described in this thesis combined with recent literature, we suggest that IKKα, in addition to its role in developmental biology (33-36), also plays an important anti-inflammatory role. This function is exerted via the negative regulation of pro-inflammatory cytokine production in macrophages (37) (38) and DC, the induction of the immunoregulatory enzyme IDO in DC and the induction of Treg by DC (39). Interestingly, these effects become particularly apparent after IKKβ inhibition. This mechanism may also have contributed to the beneficial effects that we observed after IKKβ blockade in arthritis, either via gene therapy or small molecule inhibitors.

In conclusion, the research described in this thesis might provide novel strategies to interfere with the vicious cycle of NF-κB activation in arthritis, leading to perpetuation of synovial inflammation and joint destruction. NF-κB blockade using gene therapy or small molecule inhibitors could provide innovative therapeutic approaches targeting the primary site of inflammation in patients with persistent arthritis activity in a small number of joints despite optimal systemic treatment, whereas immunotherapy with NF-κB-blocked DC could be a sophisticated yet powerful new treatment for autoimmune diseases like RA. Achievement of this goal will have a substantial beneficial effect on the RA patient and ultimately on society.


Nederlandse samenvatting
ACHTERGROND

Reumatoïde artritis (RA) is een chronische ontstekingsziekte van de gewrichten waarbij vooral het weefsel dat het gewricht aan de binnenkant bekleedt, de zgn. synoviale membraan, is aangedaan (synovitis). De belangrijkste verschijnselen zijn pijn, rode gezwollen gewrichten en bewegingsbeperking (artritis). RA komt voor bij ca. 1% van de bevolking, drie keer meer bij vrouwen dan bij mannen, en openbaart zich meestal tussen het 40e en 70e levensjaar. Bij sommige patiënten is het beloop betrekkelijk mild, maar in de meeste gevallen is er sprake van ernstige schade aan de gewrichten, leidend tot forse beperkingen. Hoewel de oorzaak van RA nog grotendeels onbekend is, kan deze ziekte de laatste jaren toch steeds beter bestreden worden. De behandeling van RA bestaat uit een snelle start met krachtige ontstekingsremmers en zgn. “disease modifying antirheumatic drugs” (DMARD’s), zoals methotrexaat. Tegenwoordig worden hieraan in toenemende mate zgn. “biologicals” toegevoegd: antilichamen of oplosbare receptoren die specifiek een bepaald ontstekingsbevorderend eiwit blokkeren. Deze behandelingen blijken zeer effectief en het is hierdoor veelal mogelijk om de onherstelbare gewrichtsschade in belangrijke mate te voorkomen. Niettemin, is er nog altijd een behoorlijk percentage patiënten dat niet reageert op deze nieuwe therapieën. Het is dan ook nodig continu onderzoek te blijven doen naar nieuwe behandelmethodes. Een nieuwe behandeling zou idealiter specifiek in het gewricht de ontsteking moeten remmen, terwijl normale functies intact blijven en de bijwerkingen minimaal zijn. Het specifiek remmen van boodschapper moleculen in de cel (intra-cellulaire signaaltransductie routes of transcriptiefactoren) die betrokken zijn bij het ontstaan en in stand houden van ontsteking lijkt daarom een krachtige manier om de pathologische (ziekmakende) cellulaire processen in artritis te bestrijden. In dit proefschrift hebben we de rol van een van deze ontstekingsbevorderende transcriptiefactoren, nuclear factor-κB (NF-κB), in RA onderzocht.

DE ROL VAN NF-κB IN REUMATOIDE ARTRITIS

Ongecontroleerde activatie van NF-κB wordt gezien als een belangrijke factor in het ontstaan van artritis. In de normale situatie wordt NF-κB inactief gehouden door de inhibitor of κB (IκB). In het geval van ontsteking vindt er fosforylatie en afbraak van IκB plaats, waardoor NF-κB geactiveerd wordt. Dit proces wordt gereguleerd door IκB kinase (IKK). Het IKK-complex bestaat uit drie componenten, IKKα, IKKβ en IKKγ/NEMO (zie schema hoofdstuk 1). Recente data laten zien dat IKKβ de belangrijkste rol speelt bij de activatie van NF-κB. In het ontstoken synoviale weefsel in de gewrichten van RA patiënten wordt hyperactivatie van NF-κB gevonden, onder andere gekenmerkt door een hoge aanwezigheid (expressie) van IKKβ in dit weefsel. Dit correleerde bovendien significant met de aanwezigheid van botschade en
de progressie van botschade na 1 jaar. Deze bevindingen suggereren dat IKKβ een centrale rol speelt in het ontstaan van de gewrichtsontsteking in RA. Theoretisch zou het blokkeren van NF-κB door het verlagen van de activiteit van IKKβ leiden tot verminderde productie van ontstekingsbevorderende eiwitten. Wij hebben dit onderzocht in een proefdiermodel voor RA in ratten en in synoviaal weefsel van RA patiënten dat d.m.v. een kijkoperatie uit het gewricht werd verkregen.

In hoofdstuk 1 wordt een inleiding gegeven waarin de huidige kennis over de oorzaken, kenmerken en gevolgen van RA uiteen worden gezet. Tevens worden twee belangrijke NF-κB activatie-routes besproken. Hoofdstuk 2 bespreekt niet alleen de huidige literatuur op het gebied van alle signaaltransductie routes die een belangrijke rol spelen in het ontstaan van RA, maar ook de manieren om deze routes (met name de NF-κB route) te blokkeren als potentiële toekomstige behandeling van RA.

**GENTHERAPIE VOOR ARTRITIS**

Gentherapie is een goede manier om bepaalde defecten in cellen langdurig te herstellen, bijvoorbeeld door deze cel te voorzien van een correcte versie van een gen of van een zgn. therapeutisch (genezend) gen dat het defect op een andere manier opheft. De op dit moment meest efficiënte gentherapie methodes maken gebruik van virussen als vector (middel) om het therapeutische gen in de cel te krijgen. Deze virussen worden in het laboratorium zodanig veranderd dat er in het virus ruimte ontstaat voor een therapeutisch gen. Vervolgens kan dit virus worden gebruikt om het therapeutisch gen te transporteren naar een defecte cel of een ontstoken gewricht met daarin ontspoorde immuuncellen die de ontstekingsreactie lokaal op gang houden. Dit gen zorgt voor een langdurige lokale productie van het therapeutische eiwit wat potentieel een gunstig effect op de artritis heeft. Er zijn vele soorten virale vectoren, ieder met voor- en nadelen.

In een eerdere studie werd het therapeutische effect beoordeeld van een gen dat een specifieke NF-κB remmer tot expressie brengt, zgn. dominant negatief IKKβ (IKKβdn). Hiertoe werd een proefdiermodel voor artritis gebruikt: rat adjuvans artritis, waarbij het gen met behulp van een adenovirus in het ontstoken gewricht werd gebracht. In hoofdstuk 3 beschrijven wij een methode om de aanwezigheid van bepaalde ontstekingsbevorderende boodschapper eiwitten (cytokines) en andere ontstekingsfactoren die een belangrijke rol spelen in de ontstekingsreactie in het synoviale weefsel van ratten te kunnen bepalen d.m.v. zgn. immunohistochemische kleuringen. Daarna werd dit gekwantificeerd middels microscopie en digitale beeld analyse. Deze techniek werd vervolgens gebruikt in de overige dierexperimentele studies beschreven in dit proefschrift. Bovendien liet deze studie zien
dat IKKβdn een sterk verminderend effect heeft op de ontstekingsreactie in het synoviale weefsel.

Hoewel adenovirale vectoren geschikt zijn om kortdurend het therapeutisch effect van een bepaald gen te beoordelen, zijn zij door bepaalde eigenschappen niet geschikt voor de langdurige behandeling van chronische ziekten zoals RA. Recente ontwikkelingen wijzen erop dat zgn. adeno-geassocieerde virusen (AAV) het meest veelbelovend zijn voor toepassing in artritis. AAV zijn onderverdeeld in verschillende serotypes, die ieder andere eigenschappen hebben waardoor ze een voorkeur hebben voor specifieke cellen en weefsels. Het ene serotype is daarom bijvoorbeeld goed om het therapeutische gen in hersencellen te krijgen, terwijl het andere serotype weer beter is om een gen over te brengen naar spierweefsel.

In hoofdstuk 4 hebben wij eerst onderzocht welk AAV serotype het best gebruikt zou kunnen worden voor lokale gentherapie bij artritis. Wederom werd het ratmodel voor artritis gebruikt. Op basis van de resultaten beschreven in dit hoofdstuk concluderen wij dat van de onderzochte serotypes 1-5, AAV serotype 5 (AAVS) het meest geschikt is om therapeutische genen in het ontstoken gewricht te krijgen. Vervolgens hebben we in hoofdstuk 5 het effect beoordeelde van NF-κB blokkade met behulp van AAVS gentherapie. Daartoe werd het eerder genoemde therapeutische IKKβdn gen m.b.v. AAVS in het ontstoken gewricht gebracht (AAVS.IKKβdn). Introductie van het IKKβdn gen resulteerde in significant verminderde gewrichtsontsteking in ratten met artritis. Bovendien hebben we in dit hoofdstuk laten zien dat het ook mogelijk is synoviaal weefsel van RA patiënten, dat d.m.v. een kijkoperatie uit het gewricht werd verkregen, in het laboratorium te behandelen met AAVS.IKKβdn. Dit leidde tot minder ontsteking in het synoviale weefsel. Hiermee slaat dit onderzoek een cruciale brug tussen toepassing van gentherapie in proefdiermodellen en het uiteindelijke doel: het behandelen van RA patiënten. Uiteraard is er nog uitgebreid aanvullend onderzoek nodig voordat deze vorm van therapie daadwerkelijk in grote groepen patiënten kan worden toegepast. Op dit moment wordt de veiligheid en effectiviteit van AAV gentherapie onderzocht in een klinische trial bij RA patiënten in de VS en Canada.

FARMACOLOGISCHE NF-κB REMMING

Hoewel gentherapie een zeer veelbelovende methode is voor de behandeling van RA, is er ook grote interesse voor farmacologische NF-κB/IKKβ remmers. Deze stoffen hebben als voordeel dat ze in pilvorm kunnen worden gegeven of met een injectie direct in het gewricht kunnen worden gebracht zonder gebruik te maken van een (virale) vector. Een belangrijk nadeel vergeleken met gentherapie is echter de vereiste herhaaldelijke toediening, omdat deze remmer niet lokaal wordt geproduceerd maar op den duur net als andere medicijnen
wordt opgeruimd door het lichaam. In hoofdstuk 6 is het therapeutisch effect van een specifieke farmacologische IKKβ remmer, het NBD peptide, beschreven. Dit NBD peptide resulteerde niet alleen in verminderde artritis, maar (op basis van röntgenfoto's) ook in minder gewrichtsdestructie. Samenvattend, hebben wij in een proefdiermodel van artritis laten zien dat IKKβ een goed target (doelwit) is om NF-κB activatie te remmen. Bovendien werd aangetoond dat remming van IKKβ resulteert in verminderde ontstekingsreacties in humane synoviale cellen en synoviaal weefsel van RA patiënten, zowel met gentherapie als met farmacologische remmers.

DE ROL VAN DENDRITISCHE CELLEN IN REUMATOIDE ARTRITIS

Bij de ontstekingsreactie die optreedt in de gewrichten van RA patiënten spelen niet alleen gewrichtscellen, maar ook immuuncellen zoals witte bloedcellen een belangrijke rol. Dendritische cellen (DC) zijn witte bloedcellen met een unieke rol in ontstekingsreacties. DC zijn uitgerust met antennes die lichaamsvreemde en lichaamseigen eiwitten kunnen onderscheiden. Vervolgens kan de DC een interactie aangaan met andere immuuncellen zoals T cellen. Deze interactie kan verschillende gevolgen hebben: 1) er ontstaat een ontstekingsreactie tegen het lichaamsvreemde eiwit door activatie van T cellen (effector T cellen). 2) DC onderdrukken de ontstekingsreacties, bijvoorbeeld indien lichaamseigen eiwitten worden herkend. T cellen worden dan geremd in hun groei en differentiatie, zodat er minder of geen ontsteking plaatsvindt (tolerantie). 3) Onder specifieke omstandigheden kunnen DC T cellen zodanig instrueren dat deze cellen vervolgens immuunreacties van andere (effector) T cellen dempen. Deze remmende “regulatoire T cellen” zijn een zeer krachtige manier om ontstekingsreacties te verminderen.

Soms ontstaat er ten onrechte een afweerreactie tegen lichaamseigen structuren, we spreken dan (zoals bij RA) van een auto-immuunziekte. DC lijken een cruciale rol te spelen in het ontstaan van dit soort aandoeningen en zijn tevens zeer belangrijk voor het in stand houden van ontstekingsreacties, zoals de synovitis in RA patiënten.

In hoofdstuk 7 werd onderzocht in hoeverre DC gevonden kunnen worden in het ontstekingsweefsel afkomstig uit gewrichten van patiënten met een gewrichtsontsteking, zoals RA. In synoviale weefsel van RA patiënten werden DC gevonden met ontstekingsbevorderende kenmerken. Bovendien werden deze DC gevonden in de directe nabijheid van T cellen, waarmee ze tijdens de ontstekingsreactie nauw contact hebben. Deze resultaten wijzen op een belangrijke rol voor DC in het op gang brengen en in stand houden van de ontstekingsreactie in artritis.
Het zou daarom nuttig zijn om de ontstekingsbevorderende functie van DC in het synoviale weefsel om te buigen naar een ontstekingsremmende functie. Ook zouden DC buiten het lichaam van de patiënt in het lab zodanig kunnen worden bewerkt dat ze ontstekingsremmende functies krijgen om vervolgens weer terug te worden gegeven aan de patiënt d.m.v. een infuus of een injectie (immunotherapie). Deze DC zouden kunnen worden gebruikt om een verscheidenheid aan autoimmuunziekten, zoals RA, te behandelen of om afstotingsreacties na orgaantransplantatie te voorkomen.

**NF-κB REMMING IN DENDRITISCHE CELLEN**

NF-κB speelt een belangrijke rol in een aantal ontstekingsbevorderende functies van DC en eerdere studies hebben laten zien dat NF-κB remming een van de beste manieren is om ontstekingsremmende DC te verkrijgen. Op basis van deze feiten hebben wij in de hoofdstukken 8-10 de effecten van NF-κB blokkade in DC bestudeerd. Daartoe werd in hoofdstuk 8 gebruik gemaakt van de NF-κB remmer “caffeic acid phenethyl ester” (CAPE). Behandeling van DC met CAPE resulteerde in blokkade van ontstekingsbevorderende factoren, zoals cytokines, en minder activatie van effector T cellen. Interessant genoeg leidde CAPE behandeling tot een verhoogde expressie van de chemokine receptor CCR7 die de DC in staat stelt zich te verplaatsen naar lymfklieren of ontstoken weefsels om daar de afweerreactie te onderdrukken.

In hoofdstuk 9 werden de gevolgen van IKKβ remming in DC geanalyseerd. Behandeling van DC met het NBD peptide leidde tot zeer specifieke remming van NF-κB activatie. In overeenstemming hiermee werd minder productie van ontstekingsbevorderende factoren en minder activatie van effector T cellen gevonden. Deze T cellen waren bovendien minder gedifferentieerd, wat gezien kan worden als tolerantie. Vervolgens werd in hoofdstuk 10 remming van IKKβ in DC (m.b.v. het NBD peptide) gecombineerd met een specifieke stimulus (CD40L). Dit resulteerde in de expressie van het "immunoregulatoire" enzym indoleamine-2,3-dioxygenase (IDO). Uiteindelijk leidde dit tot het ontstaan van regulatoire T cellen die in staat zijn geactiveerde ontstekingsbevorderende T cellen in hun functie te remmen. Deze resultaten maken duidelijk dat de effecten van specifieke IKKβ remming in DC gebruikt kunnen worden in immunotherapie op basis van DC met als doel de ontstekingsreactie te remmen bij transplantatie, auto-immuunziekten zoals RA, en allergieën.

Hoofdstuk 11 plaatst de bevindingen van de hoofdstukken 3 tot en met 10 in een breder perspectief, waarbij de nadruk wordt gelegd op de belangrijke ontstekingsbevorderende rol van IKKβ in ontstekingsreacties en de ontstekingsremmende rol van IKKα in dit soort reacties. Tenslotte wordt op basis van recente literatuur en resultaten uit dit proefschrift een model samengesteld voor de rol van IKKα en IKKβ in ontsteking.
NAWOORD

Uiteraard zou het werk beschreven in dit proefschrift er heel anders hebben uitgezien zonder de inzet van veel mensen, die ik daarvoor erg dankbaar ben. Ik wil een aantal mensen in het bijzonder bedanken.

Paul-Peter Tak, ik ben je zeer erkentelijk voor de geboden vrijheid en mogelijkheden om in mijn promotieonderzoek interessante onverwachte bevindingen verder te onderzoeken, ook al viel dat soms buiten de directe scoop van het project. Ik heb grote bewondering voor de manier waarop je in korte tijd van de Klinische Immunologie en Reumatologie (KIR) een grote en bruisende afdeling hebt gemaakt, met een goede integratie van kliniek en laboratorium. Bovendien vind ik het verheugend dat het ontwikkelen van gentherapie voor RA wordt voortgezet in de spin-off van onze afdeling, het bedrijf Arthrogen.

Margriet Vervoordeldonk, ik heb onze goede samenwerking enorm gewaardeerd. Verder ben je niet alleen een expert op het gebied van gentherapie, maar ook een erg gezellige begeleider.

Ik wil Esther de Jong bedanken voor de productieve en plezierige samenwerking in het DC onderzoek, maar bovendien voor het enthousiasme waarmee je dat doet. In een latere fase was ook Martien Kapsenberg nauw betrokken bij dit werk en ik kijk met heel veel plezier terug op onze gezamenlijke (lange) wetenschappelijke discussies. Ik vond het fantastisch om op jullie afdeling met deskundige en vooral ook gezellige mensen te mogen werken.

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I have been very fortunate to collaborate with Drs. Michael May and Sankar Ghosh, two well-known experts in NF-κB biology, who provided us with the unique NBD peptides and contributed significantly to this thesis through discussing the results.

Mijn ouders wil ik danken voor hun liefde, steun en interesse in alles wat ik doe. Bovendien vind ik het fanTAStisch dat ze mij, Ruben en Maarten altijd vrijheid en kansen bieden om onze interesses te ontwikkelen of onze dromen na te streven.

Tenslotte een speciaal woord van dank voor Simone “because two hearts are better than one”. Bovendien blijkt nu Dennis er is, dat 3 nog mooier is!
CURRICULUM VITAE

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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AA</td>
<td>adjuvant arthritis</td>
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<td>Ad</td>
<td>adenovirus</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
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<tr>
<td>DC</td>
<td>dendritic cell(s)</td>
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<tr>
<td>DMARD</td>
<td>disease-modifying anti-rheumatic drug</td>
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<tr>
<td>dn</td>
<td>dominant negative</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DPI</td>
<td>diphenylene iodoniumchloride</td>
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<td>FasL</td>
<td>Fas ligand</td>
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<td>FLIP</td>
<td>Fas-associated death domain-like IL-1β -converting enzyme-inhibitory protein</td>
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<td>i.a.</td>
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<td>intercellular adhesion molecule</td>
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<td>indoleamine 2,3-dioxygenase</td>
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<td>IκB kinase</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>IOD</td>
<td>integrated optical density</td>
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<td>ISRE</td>
<td>interferon stimulated response element</td>
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<tr>
<td>IkB</td>
<td>inhibitor of kB</td>
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<td>MALT1</td>
<td>mucosa associated lymphoid tissue lymphoma translocation gene 1</td>
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<td>mitogen-activated protein kinase</td>
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<td>MAPK-activated protein</td>
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<td>MAPK kinase</td>
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<td>monocyte chemotactic protein</td>
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<td>transformed 3T3 cell double minute 2, p53 binding protein</td>
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<td>mitogen-activated ERK-activating kinase</td>
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<td>mixed lymphocyte reaction</td>
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<td>matrix metalloproteinase</td>
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<td>molecular target of rapamycin</td>
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<td>nuclear factor of activated T cells</td>
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<td>natural killer</td>
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<td>nuclear localization sequence</td>
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<td>non-steroidal anti-inflammatory drug</td>
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<td>protein tyrosine kinase</td>
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<td>rheumatoid arthritis</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>rAAV</td>
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<td>Staphylococcus enterotoxin B</td>
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<td>Src-homology 2 (SH-2)-domain-containing inositol polyphosphate phosphatases</td>
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<td>suppressor of cytokine signaling</td>
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<td>signal transducer and activator of transcription</td>
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<td>T helper 1</td>
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<td>tissue inhibitor of MMPs</td>
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<td>tumor necrosis factor α</td>
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<td>TNF receptor-associated factor</td>
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<tr>
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<td>TRIF-related adapter molecule</td>
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<td>regulatory T cell</td>
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<td>TIR-domain-containing adapter-inducing interferon-beta</td>
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<td>vascular cell adhesion molecule</td>
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<tr>
<td>ZAP-70</td>
<td>ζ-associated polypeptide of 70kDa</td>
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Chapter 1. Figure 2. IKKβ and phIKBα expression in human RA synovial tissue.
(A) Representative picture of RA synovial tissue negative control (H&E staining). (B) Representative picture of RA synovial tissue showing IKKβ staining. (C) Representative picture of RA synovial tissue showing phosphorylated (ph)IKBα staining. (D) Significant correlation between IKKβ and phIKBα expression in RA synovial tissue (n=15; *p* < 0.05; IOD = integrated optical density; J. Ludikhuize et al. Manuscript in preparation)
Chapter 3. Figure 1. Reduced synovial pro-inflammatory cytokine and MMP3 expression in Ad.IKKβdn treated rats.

Expression of IL-1β, TNFα, IL-6, IL-10, MMP3 and TIMP-1 in arthritic ankle joints of rats treated with Ad.IKKβdn. Representative pictures of synovial cytokine staining are shown (overview + detailed image of TNFα expression), followed by results from digital image analysis of the immunohistochemical stainings (n=10/group). Data are expressed as IOD ± SEM (* P < 0.05).
Chapter 4. Figure 1. β-gal expression in rat synovial tissue 2 weeks after i.a. injection of different rAAV serotypes.

Joints were snap frozen and sections were cut and stained in situ for β-Gal activity and counterstained with nuclear red. Representative cryosections of right ankle joints of rats with AA, injected with $6.1 \times 10^{10}$ GC rAAV, Ad.LacZ as a positive control or saline as a negative control are shown (magnification 100x, 400x). The lining layer is indicated by arrows (A). Tissue sections were quantified for β-Gal expression by computer assisted digital image analysis (B). Values are expressed as cumulated IOD per mm² (IOD: Integrated Optical Density) +/- s.d. * Statistically different using the Mann-Whitney U test with P-values <0.05 as compared to the control group.
Chapter 4. Figure 6. Transduction of fibroblast-like synoviocytes (FLS) with rAAV in vitro.

Human FLS isolated from synovial biopsies from RA patients were incubated with rAAV2.GFP or rAAV5.GFP. After forty-eight hours of incubation, the cells were fixed and transgene expression was determined by fluorescent microscopy. Representative pictures of three independent experiments using cells from three different patients are shown. Right: fluorescent microscopy, left: phase contrast microscopy.
Chapter 5. Figure 1. AAV5IKKβdn blocks inflammatory responses in human macrophages and RA FLS in vitro.

(A) Fibroblast-like synoviocytes (FLS) were transduced with either AAV5.IKKβdn or AAV5.eGFP at an MOI of 1000. After 3 days GFP expression was evaluated using fluorescent microscopy. Representative pictures are shown. (B) Transduced FLS were stimulated with IL-1β (2.5 ng/ml) for 30 min, extensively washed and lysed in sample buffer. Cell lysates were analyzed by western blotting. IL-1β-induced IkBα phosphorylation was blocked after AAV5.IKKβdn transduction as demonstrated by quantification of the blots by densitometry. One representative experiment out of three is shown. (C) AAV5 is capable of transducing human macrophages. Macrophages were transduced with AAV5.eGFP at an MOI of 1000 and after 3 days GFP expression was evaluated. Representative light and fluorescent microscopy pictures are shown. (D) AAV5.IKKβdn blocks IL-1β induced TNFα production by human macrophages. Macrophages were transduced with either AAV5.IKKβdn or AAV5.eGFP at an MOI of 1000. After 3 days, cells were stimulated with IL-1β (2.5 ng/ml) and after 24 h TNFα levels were measured in the supernatant by ELISA. Data represent three independent experiments performed in triplicates.
Chapter 5. Figure 3. Reduced pro-inflammatory cytokine expression in AAV5.IKKβdn treated rats.

(A) Expression of pro-inflammatory cytokines in arthritic ankle joints of rats treated with AAV5.IKKβdn on day 10. Representative pictures (overview + detailed image) of synovial cytokine staining are shown, followed by results from digital image analysis of the immunohistochemical stainings (n=10/group; mean ± SEM). TNFα and IL-6 expression was significantly reduced in the AAV5.IKKβdn treated group (p=0.03 and p=0.005 respectively). IL-10 expression was not altered by AAV5.IKKβdn treatment. (B) Expression of pro-inflammatory cytokines in arthritic ankle joints of rats treated with AAV5.IKKβdn on day 6. Results from digital image analysis of immunohistochemical stainings are shown (n=10/group; mean ± SEM). No significant differences in TNFα, IL-6 and IL-10 expression were observed between the groups.
Chapter 5. Figure 5. AAV5.IKKβdn blocks TNFα-induced IL-6 production of RA synovial biopsies ex vivo.

(A) Immunohistochemical detection of GFP in synovial biopsies 3 days after transduction with AAV5.IKKβdn or AAV5.eGFP (2.5x10^10 GC/ml). (B) Transduced biopsies were subsequently stimulated with TNFα (10 ng/ml) for another 4 days. At day 7, supernatants were collected and IL-6 levels were measured by sandwich ELISA. AAV5.IKKβdn treatment of RA synovial tissue resulted in significantly reduced IL-6 production (p=0.03). Data are representative of three independent experiments performed in triplicates.
Chapter 6. Figure 3. Intra-articular NBD treatment results in decreased pro-inflammatory cytokine expression.

Shown are representative overview pictures of NBD or MUT treated ankle joints. Expression of different cytokines was evaluated by immunohistochemical staining of paraffin embedded ankle joints. Detailed images of synovial cytokine staining are provided (boxes in upper panels indicate synovial tissue location), followed by results from digital image analysis (n=10/group). TNFα and IL-1β expression was significantly reduced in the NBD treated group, compared to the MUT treated group (* P = 0.05 and ** P = 0.04 respectively). Data represent mean ± SEM.
Chapter 7. Figure 1. Expression of CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC in synovial tissue from patients with RA, OA and PsA.

(A) Representative sections of synovial tissue are shown (original magnification x 400). (B) No significant differences were observed between the numbers of both mDC and pDC present in RA, PsA and inflammatory OA synovia. (C) In RA, PsA, and inflammatory OA synovia the numbers of pDC are significant higher compared to mDC. In (B) and (C) sections were analyzed using computer assisted digital image analysis. Results are shown as mean numbers of positive cells/mm²±SEM of 22 patients with RA, 10 patients with PsA and 11 patients with inflammatory OA. Differences between two groups were analyzed for statistical significance with the Mann-Whitney U test (*P <0.05, **P <0.01, ***P <0.001).

Chapter 7. Figure 2. Phenotype of mDC and pDC in RA synovium.

Double immunohistochemistry stainings were performed to investigate the coexpression of CD1c (myeloid marker) or CD123 with CD1c (BDCA1) and CD304 (BDCA4), respectively. CD1c (BDCA1)+ mDC coexpress CD11c (blue) and CD304 (BDCA4)+ pDC coexpress CD123 (blue). A representative double immunostaining of RA synovium from one patient is shown. (Original magnification x400).
Chapter 7. Figure 3. Maturation status of mDC and pDC in RA, PsA and inflammatory OA synovia.

(A) The percentage of both mDC and pDC coexpressing the DC maturation marker CD83 in RA synovium did not differ significantly compared to PsA and inflammatory OA synovia. (B) A representative double immunofluorescence staining of RA synovium from one patient is shown. CD1c (BDCA1)+ and CD303 (BDCA2)+ DC in green, CD83+ cells in red and double positive cells in yellow (Original magnification x400).

Chapter 7. Figure 4. mDC and pDC are localized in lymphocyte aggregates in RA synovial tissue.

Double immunohistochemistry stainings were performed to investigate the distribution of CD1c (BDCA1)+ mDC and CD304 (BDCA4)+ pDC in relation with CD3 and CD8 positive T cells. Both mDC and pDC in RA synovium can be identified in close proximity to clusters of CD3 and CD8 positive cells. A representative double immunostaining of RA synovium from one patient is shown. (Original magnification x400).
Chapter 7. Figure 5. Expression of cytokines by mDC and pDC in RA synovium.

Double immunofluorescence stainings were performed to investigate the expression of IL-12p70, IL-15, IL-18, IL-23p19, IFN-α or IFN-β by CD1c (BDCA1)+ mDC and CD303 (BDCA2)+ pDC. (A) A representative double immunofluorescence staining of RA synovium from one patient is shown. CD1c (BDCA1)+ and CD303 (BDCA2)+ DC in green, cytokines in red and double positive cells in yellow (Original magnification x400). (B) Quantification of cytokine expression by mDC and pDC. Differences between two groups were analyzed for statistical significance with the Mann-Whitney U test (*P<0.05, **P<0.01, ***P<0.001).
Chapter 8. Figure 2. CAPE blocks LPS-induced NF-κB activation.

(A) CAPE blocks LPS-induced IκBα phosphorylation. Monocyte-derived DC were pre-incubated with either medium or CAPE at a concentration of 10 µg/ml for 2 h. Subsequently, cells were stimulated with 200 ng/ml LPS for 30 min, extensively washed and lysed in sample buffer. Cell lysates were analyzed by western blotting and densitometry was performed. One representative experiment out of three is shown; densitometry includes data from all experiments (*p<0.05).

(B) CAPE blocks nuclear translocation of p65 and MHC class II surface expression. Series of confocal images of monocyte-derived DC cultured on glass chamber slides and stimulated for 4 h with LPS (200 ng/ml) in the presence or absence of CAPE or controls. Cells were fixed in cold acetone and stained for p65 and HLA-DR expression. Nuclei were stained with Hoechst and cells were analyzed by scanning the entire cell using a confocal laser microscope. In the overlay picture nuclear translocation can be evaluated. Representative pictures from one experiment are shown. Results are representative for three independent experiments.
Chapter 9. Figure 2. NBD peptide blocks nuclear translocation of p65 and MHC class II surface expression.

Series of confocal images of monocyte-derived DC cultured on glass chamber slides and stimulated for 4 h with LPS (200 ng/ml) in the presence or absence of NBD/MUT peptides (50 μM) or controls. Cells were fixed in cold acetone and stained for p65 and HLA-DR expression as described in the Methods section. Nuclei were stained with Hoechst and cells were analyzed by scanning the entire cell using a confocal laser microscope. In the overlay picture nuclear translocation can be evaluated. Representative pictures from one experiment are shown. Results are representative for three independent experiments.
Chapter 10. Figure 2. NBD peptide selectively blocks canonical NF-κB activation

(A) NBD peptide blocks CD40L induced IκBα phosphorylation. Monocyte-derived DC were pre-incubated with either NBD peptide or MUT peptide for 2 h. Subsequently, cells were stimulated with CD40L for 30 min, extensively washed and lysed in sample buffer. Cell lysates were analyzed by western blotting and densitometry was performed. One representative experiment out of three is shown; densitometry includes data from all experiments (*p < 0.05). (B) NBD peptide completely blocks nuclear translocation of RelB following LPS stimulation, whereas CD40L induced RelB translocation is only marginally affected. Series of confocal images of monocyte-derived DC stimulated for 4 h with LPS or CD40L in the presence or absence of NBD/MUT peptides or controls. Cells were centrifuged onto glass slides, fixed in cold acetone and stained for RelB expression. Nuclei were stained with Hoechst and cells were analyzed by scanning the entire cell using a confocal laser microscope. In the displayed overlay pictures RelB nuclear translocation can be evaluated. Representative pictures from one experiment are shown. Results are representative for three independent experiments.