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THE ROLE OF NF-κB IN INFLAMMATORY DISEASES

1. MOLECULAR AND CELLULAR BASIS OF INFLAMMATION AND ITS ROLE IN PATHOLOGY

The term inflammation was used as early as 1600 BC in Egyptian papyrus writings to describe a complex series of events induced by a wound or an infection. These events can be recognized by the redness, swelling, fever and pain observed, and are now known to reflect the vasodilation, increased capillary permeability and influx of phagocytic cells. Thus, under most circumstances, inflammation is a normal physiological and very beneficial response, employed by our organism to fight infectious agents and protect us from disease. Inflammation involves the activation of both the innate and adaptive branches of the immune system and is under the control of a large number of cellular and molecular components. Major cellular components include B-cells, T-cells, dendritic cells and macrophages (Table 1).

Major molecular components involved in inflammation comprise cytokines, adhesion molecules, acute phase proteins, kinins, prostaglandins/leukotrienes, nitric oxide (NO) and inducible NO synthase (iNOS), and histamine. Cytokines are small proteins or glycoproteins that act as local messengers in inflammation and immunity, but also in many other important biological processes such as cell growth, development, repair and fibrosis. Cytokines are considered to play a central role in the development of inflammatory responses, as they regulate both the recruitment and activation of the cellular components, as well as the expression of the molecular components of inflammation. Their own expression, in turn, is subject to control by the other molecular components, thus setting up a regulatory loop that can either reduce or extend the persistence of the inflammatory response. Adhesion molecules are essential for the adhesion, activation and transmigration of leukocytes to inflammatory sites as well as the costimulation and activation process of T- and B-cells upon recognition of antigen (Ley, 2001). Acute phase proteins are serum proteins released in response to tissue damage. C-reactive protein, in particular, is produced by the liver and binds to the C-polysaccharide cell wall component of bacteria and fungi, activating complement, mediating phagocytosis and increasing pathogen clearance (Mortensen, 2001).

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Kinins are small peptides, found in blood plasma in an inactive form, that become activated in response to tissue injury to cause vasodilation and increased capillary permeability (Couture et al., 2001). Some kinins such as bradykinin, also stimulate pain receptors in the skin. Finally, prostaglandins and leukotrienes are eicosanoid lipid mediators derived from phospholipase-released arachidonic acid that are involved in numerous homeostatic and inflammatory functions, including vasodilation, chemotaxis, fever and pain (Funk, 2001). NO is also a mediator of inflammation that is important in bronchodilation, leukocyte adhesion and extravasation to tissues and antimicrobial activity through its oxidant derivatives (Bogdan, 2001). Production of NO is under the control of two constitutive NO synthase enzymes and one inducible NO synthase enzyme, which renders its study very difficult.

Table 1. Major functions of immune system cells.

Cell type		*Function*
Lymphoid	Helper T-lymphocytes	Stimulate B-lymphocyte growth and differentiation, and activate macrophages
	Cytotoxic T-lymphocytes	Kill virally infected or tumor cells and activate macrophages
	B-lymphocytes	Present antigen to T-lymphocytes and produce antibodies
	Large granular lymphocytes or natural killer (NK) cells	Involved in the early responses to virally infected or tumor cells and mediate antibody-dependent cellular cytotoxicity
Myeloid	Mononuclear phagocytes (monocytes/macrophages)	Phagocytose foreign particles and dead cells, present antigen to T-lymphocytes, induce fibroblast and vascular endothelial cell proliferation important in tissue repair, and recruit other inflammatory cells
	Neutrophils	Phagocytose foreign particles and dead cells
	Eosinophils	Phagocytose IgE-coated particles and mediate immediate-type hypersensitivity reactions
	Basophils/mast cells	Involved in IgE-mediated hypersensitivity
	Platelets	Mediate blood clotting
	Dendritic cells	Present antigen to T-lymphocytes
Endothelial		Regulate the recruitment of effector cells and the blood-clotting process

Although inflammation is a very beneficial response, prolonged or deregulated inflammation can lead to extensive tissue damage, disability or even death. A number of human diseases have been attributed to sustained inflammatory responses and include rheumatoid arthritis, inflammatory bowel disease, inflammatory lung diseases (asthma and fibrosing alveolitis), inflammatory skin diseases (psoriasis and incontinentia pigmenti), atherosclerosis, diabetes and neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis. Understanding

what regulates the inflammatory response in these conditions may lead to novel therapeutics of increased efficacy and reduced toxicity.

2. NF-κB IN THE CONTROL OF MOLECULAR FACTORS OF INFLAMMATION

In the past, most studies of inflammation were focusing on the role of mediators, such as cytokines, adhesion molecules, kinins and prostaglandins, in the development of the inflammatory response. However, in recent years, the development of powerful molecular biology tools has made it possible to study not only how distinct mediators regulate each other, but also which signaling pathways and transcription factors are involved and how they interact with each other. Central to the development of the inflammatory response was found to be the transcription factor NF-κB.

Table 2. Genes involved in inflammation and regulated by NF-κB.

Function	*Gene*
Activation of immune cells and inflammation	TNFα, IL-1, IL-2, IL-6, IL-12, IL-17, IL-18, IFNγ, GM-CSF
Chemotaxis/recruitment of immune cells	IL-8, MIP-1α/β, MCP-1, Gro-α/β/γ, eotaxin, ICAM-1, VCAM-1, E-selectin
Angiogenesis/neovascularization	VEGF
Vasodilation/increased capillary permeability	B1 kinin receptor, iNOS, COX-2, PGE2, PLA_2
Tissue damage	C-reactive protein, hsp32, hsp70
Tissue remodeling	MMP-1, MMP-3, MMP-9, MMP-13
Survival and proliferation	TRAF1/2, c-IAP1/2, XIAP, A1/Bfl-1, bcl-x_L, c-Myc, cyclin D

NF-κB is essential for host defense and inflammatory responses to microbial and viral infections (Barnes & Karin, 1997; Ghosh et al., 1998). Mouse models deficient in different NF-κB subunits have demonstrated the pivotal role that NF-κB plays in lymphoid organ development, immunity and inflammation (Beg et al., 1995; Sha et al., 1995; Weih et al., 1997; Franzoso et al., 1998). In response to pathogens, coordinated activation of NF-κB occurs in almost every cell type known and includes cells involved in immunity such as neutrophils, macrophages, lymphocytes, endothelial cells and epithelial cells. NF-κB activation, in turn, induces the expression of numerous inflammatory mediators such as cytokines, adhesion molecules, chemokines, cyclooxygenase 2 (COX-2), nitric oxide and others that contain NF-κB-binding sites in their promoters (Table 2) (Pahl, 1999). As many of these inflammatory mediators such as TNFα and IL-1 can also activate NF-κB, a positive regulatory loop is created that maintains the activation of NF-κB and amplifies the inflammatory response (Karin, 1999). NF-κB is tightly regulated and provides an important mechanism by which the fine balance

between beneficial immune responses to fight infections and pathological immune responses that lead to tissue damage are achieved. Here possible mechanisms of highly specific NF-κB-dependent regulation of gene expression during inflammation are discussed.

Homodimers and heterodimers of five distinct Rel proteins form the family of transcription factors collectively referred to as NF-κB. All five proteins are characterized by the presence of a common Rel homology domain responsible for DNA binding and dimerization. Nevertheless there are two distinct classes within the family. The first consists of p65, RelB and c-Rel proteins that are synthesized as mature proteins and encompass the 'transactivating domain'. The second group includes p50 and p52 proteins that first are synthesized as large precursors p105 and p100, respectively, and then undergo proteolytic processing. Neither p50 nor p52 protein contain the transactivating domain. This diversity of NF-κB complexes lays the first basis for specificity of gene regulation. Indeed, there is growing body of evidence that the p50/p50 homodimer (lacking transactivating potential) exerts an inhibitory effect on gene transcription (Schmitz & Baeuerle, 1991; Franzoso et al., 1992; Franzoso et al., 1993; Udalova et al., 2000), possibly due to a competition with p50/p65 for binding to DNA. Overexpression of p50 blocked lipopolysaccharide (LPS)-induced transcription from TNFα promoter reporter constructs, suggesting that this factor is an inhibitor of the TNFα gene (Baer et al., 1998).

It has been also observed that expression of IFNβ, which is transcriptionally regulated by NF-κB in response to a viral infection, is increased in p50 knockout mice (Sha et al., 1995). Which NF-κB dimer is more likely to interact with a given promoter depends both on cell-specific protein distribution and DNA-binding specificity of different NF-κB sites, which significantly differ between the dimers (Kunsch et al., 1992; Udalova et al., 2002a).

p65 and c-Rel-containing NF-κB dimers are held in the cytoplasm in an inactive form by specific inhibitors, termed as inhibitors of NF-κB (IκB) proteins such as IκBα, IκBβ and IκBε, which undergo rapid ubiquitin-dependent degradation after the cell becomes exposed to a stimulus. Tissue-specific distribution of IκB molecules together with their different preference for NF-κB dimers may constitute another level of fine gene regulation by virtue of inhibiting specific NF-κB subsets in different cells. Various IκB proteins target different NF-κB species: IκBα and IκBβ bind preferentially to p50/p65 and p50/c-Rel heterodimers (Thompson et al., 1995b), IκBε associates only with homo- or heterodimeric complexes containing p65 and c-Rel (Simeonidis et al., 1997; Whiteside et al., 1997), and Bcl-3, another member of the IκB family, interacts specifically with p50 and p52 homodimers (Franzoso et al., 1992; Wulczyn et al., 1992). Bcl-3 can form ternary complexes with DNA-bound p52/p52, and enables it to activate transcription directly (Bours et al., 1993; Fujita et al., 1993), while interaction of this unusual member of the IκB family with DNA-bound p50/p50 is believed to induce its dissociation from DNA, allowing other NF-κB species to bind instead (Franzoso et al., 1992; Franzoso et al., 1993; Pan & McEver,

1995). Direct phosphorylation of Rel proteins constitutes another mode of NF-κB regulation (Schmitz et al., 2001). It has been suggested that Rel phosphorylation is critical for full NF-κB activation and that this can occur independently of IκB degradation. Both protein kinase A (PKA)-mediated and casein kinase II (CKII)-mediated phosphorylation of p65 have been demonstrated (Zhong et al., 1997; Wang et al., 2000). Such integration of different signaling pathways allows for activation of distinct Rel proteins to take place in response to different proinflammatory signals.

Upstream kinase cascades that result in IκB phosphorylation and degradation constitute another level of control of NF-κB activation as their activity is tightly regulated. Central to this cascade is the IκB kinase complex (IKK), a large multisubunit kinase complex that comprises IKKα, IKKβ and IKKγ, and that is the point of convergence of multiple signals (e.g. microbial and viral products, cytokines and T-cell receptor engagement) leading to IκB phosphorylation and NF-κB activation (Karin, 1999). The spectrum of adaptor proteins and kinases that signal downstream from the receptors to the IKK complex differ between microbial (Toll-like receptor), cytokine (e.g. TNF receptors) and T-cell receptor-signaling pathways. Many of the signaling molecules in these pathways are yet to be identified. However, recent studies identified a common molecule, namely receptor-interacting protein kinase (RIP1/RIP2), which is involved in signaling from Toll-like, T-cell and TNF receptors, thus integrating signals for both innate and adaptive immune responses during the inflammation (Fig. 1) (Kelliher et al., 1998; Chin et al., 2002; Kobayashi et al., 2002).

A different mechanism of NF-κB RelB/p52 translocation into the nucleus has recently emerged. RelB is associated with p100 precursor in nonstimulated cells. p105 and p100-containing complexes are held in the cytoplasm by the C-terminal IκB-like domain of the proteins, which is proteolytically processed by ubiquitin-dependent partial degradation (Karin & Ben-Neriah, 2000). Whether the processing of p105 to p50 is inducible remains in question (Belich et al., 1999; Ciechanover et al., 2001; Dumitru et al., 2000). However, there is clear evidence for regulated processing of p100 (Senftleben et al., 2001; Xiao et al., 2001). Lymphotoxins α and β (LTα, LTβ) are essential in both lymphoid organ development and inflammation (Ruddle, 1999). Signaling from the LTβ receptor (LTβR) activates the MAP3K protein NIK, which in turn activates IKKα, leading to phosphorylation, ubiquitination and degradation of the C-terminal domain of p100. While the canonical NF-κB pathway, which is essential for innate immunity (Senftleben et al., 2001), is mostly dependent on IKKβ (Li et al., 1999a-b), this alternative pathway depends on IKKα and is mostly involved in lymphoid organ development and adaptive immunity (Senftleben et al., 2001). At the same time, signaling through the LTβR has also been shown to potentially use IKKβ to induce IκBα phosphorylation and NF-κB activation (Smith et al., 2001). It remains to be determined whether two different NF-κB pathways activate distinct subsets of genes and whether the proinflammatory genes are likely to be regulated by one selected pathway.

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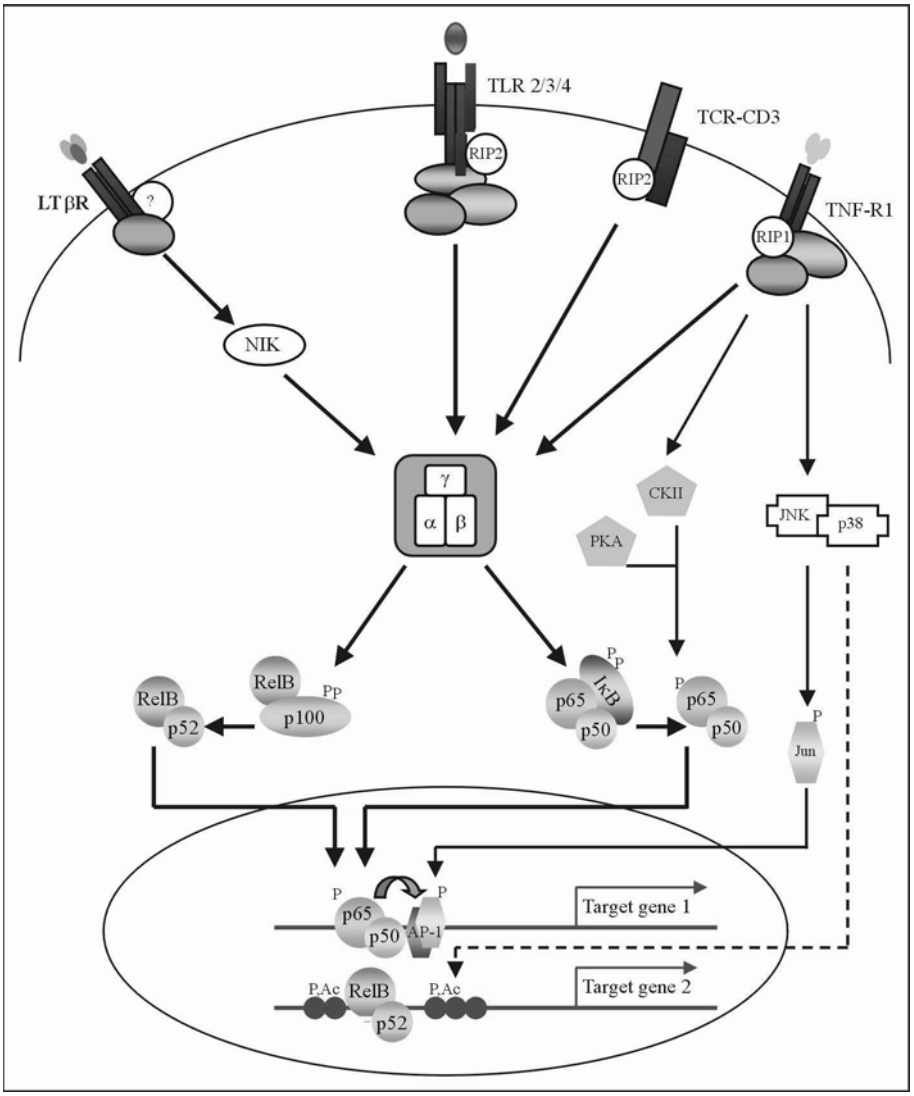

Fig. 1. Integration and specificity in NF-κB-dependent activation of the proinflammatory genes. In this model different proinflammatory stimuli are recognized by different receptors at the cell surface: TLR (for microbial lipoproteins, LPS, dsRNA), TNF-R1 and LTβR (for cytokines TNFα and LTαLTβ$_2$ trimer) and TCR (for T-cell-specific signaling). They

activate intracellular signaling cascades through distinct sets of adaptor proteins and kinases, with RIP1/2 integrating signals between the cascades. The IKK complex congregates the pathways. The activated IKK complex phosphorylates IκB, mostly by IKKβ, leading to IκB ubiquitination and degradation and the release of free NF-κB p50/p65. Its translocation into the nucleus is accompanied by PKA or CKII-dependent phosphorylation of p65. LTβR-NIK signaling is the only example to date of acting through IKKα subunit of the IKK, resulting in phosphorylation and degradation of p100 and nuclear translocation of NF-κB RelB/p52. In the nucleus p50/p65 and RelB/p52 complexes (and other NF-κB/Rel dimers not described in this simplified model) find specific DNA-binding sites in the promoters of inflammatory mediators (cytokines, adhesion molecules, nitric oxide, macrophage proteins, etc.) based on DNA sequence preferences, association with other transcription factors (e.g. AP-1) and high-order chromatin structure. The chromatin structure of the promoters can either be immediately accessible to NF-κB binding (e.g. the TNFα promoter) or require stimulus-dependent modification of histones (e.g. p38-dependent phosphorylation and acetylation of H3 histone in the IL-6 promoter). Binding of a specific NF-κB complex and its interaction with promoter-specific transcription factors leads to recruitment of coactivators and chromatin remodeling factors and results in highly specific signal-dependent activation of inflammatory gene transcription.

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Another mode of specificity in NF-κB-dependent gene activation lies in its ability to orchestrate gene expression in concert with other transcription factors. Eukaryotic genes are regulated largely through the cooperative interactions of distinct transcription factors and their assembly into multicomponent enhancer complexes (Carey, 1998). The repertoire of proteins NF-κB interacts with determines the timing, level and duration of gene expression. For instance, the production of IFNβ in response to virus induction requires the correct assembly of NF-κB p50/p65, ATF-2, c-Jun, IRF-3, IRF-7 and HMG-I(Y) into enhanceosome (Thanos & Maniatis, 1995), in which both the composition and the orientation of transcription complexes are essential (Thanos & Maniatis, 1995; Falvo et al.,

2000). Moreover, such assembly leads to ordered recruitment of coactivator molecules, histone acetylases and chromatin remodeling factors and general transcription machinery, that determines activation time and duration of the gene transcription (Agalioti et al., 2000). DNA-binding studies reveal a requirement for NF-κB and a small group of other transcriptional activators for maximal levels of adhesion molecules during inflammatory responses. The organization of the cytokine-inducible element in the E-selectin promoter is remarkably similar to the IFNβ gene in that both promoters require NF-κB, ATF-2 and HMG-I(Y) (Whitley et al., 1994), whereas vascular cell adhesion molecule-1 (VCAM-1) expression is induced through interactions of NF-κB with IRF-1 and HMG-I(Y), which also depends on constitutively present SP-1 (Neish et al., 1995a-b). NF-κB physically associates with the transcription factor NF-IL-6 in synergistic activation of transcription of the inflammatory cytokine IL-6 (Matsusaka et al., 1993), and together with AP-1 of IL-8 (Roebuck, 1999).

The ability of NF-κB to physically interact with another ubiquitous transcription factor AP-1, whose transcriptional induction follows a distinct signal transduction pathway involving phosphorylation of c-Jun kinase (JNK) (Chang & Karin, 2001), is of particular importance, as it may play a general role in regulation of the inflammatory response by simultaneous activation of the majority of inflammatory mediators (Stein et al., 1993). Indeed, many of the inflammatory genes require these two transcription factors working cooperatively, including VCAM-1 (Ahmad et al., 1998), IL-8 (Roebuck, 1999), COX-2 (Allport et al., 2000), macrophage chemoattractant protein-1 (MCP-1) (Martin et al., 1997) and collagenase-3 (Mengshol et al., 2000).

Both the extensive histone-DNA contacts in individual nucleosomes and the folding of the nucleosomal chain into higher order chromatin structures limit the accessibility of the genome to DNA-binding proteins, including NF-κB. It has been proposed that stimulation with microbial products or proinflammatory cytokines may result in two distinct waves of NF-κB recruitment to target promoters: a fast recruitment to constitutively and immediately accessible promoters (e.g. the TNFα promoter) and a late recruitment to promoters requiring stimulus-dependent modifications in chromatin structure to make NF-κB sites accessible (Saccani et al., 2001). The later recruitment was shown to selectively occur on the promoters of a subset of cytokine and chemokine genes, including IL-6, IL-8 and MCP-1, through p38 MAP kinase–dependent phosphorylation and phosphoacetylation of histone H3 (Saccani et al., 2002). p38 is not a general modulator of NF-κB recruitment to chromatin as other cryptic NF-κB sites, e.g. in the macrophage inflammatory protein-1 (MIP-1) promoter, are switched into an accessible state in a p38-independent manner. A highly promoter-specific facilitation of NF-κB recruitment to selected binding sites introduces a novel mechanism of gene activation specificity during inflammatory responses.

The mechanism of gene expression optimization that has received relatively little attention so far is the effect of genetic variations on the level of response to NF-κB. A naturally occurring polymorphism at nucleotide -863 in the human

TNFα promoter region provided an opportunity to dissect the functional NF-κB interactions at a single binding site (Udalova et al., 2000). It was found that this site normally binds both p50/p65 and p50/p50 NF-κB complexes, but a single base change specifically inhibited p50/p50 binding. Reporter gene analysis indicated that the variant allele acts to elevate inducible TNFα production. This is of fundamental interest, as it raises the question of how the genetic background may influence the immune gene regulation and consequently result in balancing of the inflammatory response. For instance, patients with severe rheumatoid arthritis, the chronic inflammatory disease in which TNFα has an essential role in pathogenesis, were twice as likely to possess the TNFα-863 allele than the control group (Udalova & Kwiatkowski, 2002). Predictions of the NF-κB-binding sites that are likely to have a functional effect were done for the human chromosome 22 and identified about fifty single nucleotide polymorphisms that would alter NF-κB binding to DNA (Udalova et al., 2002b). Together with a number of other observations of functional consequences of nucleotide polymorphisms that alter protein-DNA interactions (Knight et al., 1999; Farzaneh-Far et al., 2001; Hohjoh & Tokunaga, 2001; Price et al., 2001; van Heel et al., 2002), this novel genetic mechanism of gene regulation may have important implications in disease pathogenesis.

3. NF-κB AND ITS ROLE IN DISEASE PATHOGENESIS

3.1. Scope

The profound effect that NF-κB has on the regulation of numerous genes involved in inflammation and the ability of many such genes to potently activate NF-κB themselves, led to the suggestion that NF-κB may also be essential for the development and/or progression of inflammatory diseases. Currently, an increasing amount of data on the role of NF-κB is being generated by studies in patients and patient tissue, and is being complemented by studies in relevant animal models.

3.2. Rheumatoid arthritis

The rheumatoid synovium is a site of active inflammation. It is infiltrated by a diverse spectrum of cells such as activated T-lymphocytes, macrophages, fibroblasts, endothelial cells and plasma cells, and is enriched with almost every cytokine and inflammatory mediator known (Feldmann & Maini, 2001). Cytokines such as TNFα and IL-1 that activate NF-κB are elevated in the synovium, and activated NF-κB has been detected in human synovial tissue from both early and later stage patients in macrophage- and fibroblast-like synoviocytes (Asahara et al., 1995; Handel et al., 1995; Marok et al., 1996). In particular, macrophage-like synoviocytes that localize in the synovial lining layer and the vascular endothelium have been shown to contain p65 and p50 NF-κB subunits in their nucleus (Handel et al., 1995). In animal models of arthritis, such as collagen-induced

arthritis in mice or adjuvant-induced arthritis, pristane-induced arthritis and streptococcal cell wall (SCW)-induced arthritis in rats, NF-κB is also activated (Tsao et al., 1997; Han et al., 1998; Miagkov et al., 1998; Palombella et al., 1998).

The activation of NF-κB has important functional consequences. Studies that we performed in dissociated synovial membrane cultures from rheumatoid arthritis patients with active disease demonstrated that overexpression of IκBα, the natural inhibitor of NF-κB, significantly reduces the production of TNFα (Foxwell et al., 1998). As TNFα is central to the inflammatory cascade that takes place in the rheumatoid synovium, and is an already established and proven therapeutic target for the reduction of disease activity, this suggested that NF-κB-blocking agents may also result in therapeutic benefit in these patients. Interestingly, inhibition of NF-κB in these cultures also inhibited the production of a large number of other proinflammatory cytokines such as IL-1β, IL-6 and IL-8, without significantly affecting the production of the anti-inflammatory cytokines IL-10 and IL-11, or the release of soluble TNF receptors (Bondeson et al., 1999). In addition, inhibition of NF-κB blocked the secretion of matrix metalloproteinases (MMPs) 1, 3 and 13 (Bondeson et al., 1999). These observations suggested that NF-κB and NF-κB-dependent gene expression are central to the production of a large number of proinflammatory, but not of anti-inflammatory mediators in the rheumatoid synovium essential for disease pathogenesis (Fig. 2). These proinflammatory mediators in turn induce NF-κB activation, thus establishing a vicious circle that maintains NF-κB activation and perpetuates inflammation.

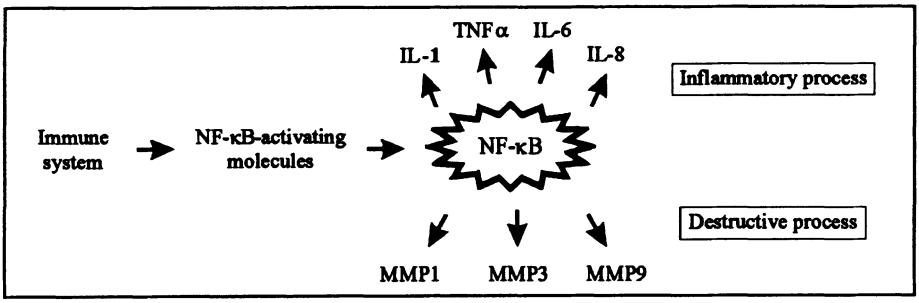

Fig. 2. NF-κB is central to the regulation of inflammatory and destructive processes in the rheumatoid synovium. Therapeutics aimed at blocking NF-κB or upstream NF-κB-activating molecules constitute a promising new approach to the treatment of rheumatoid arthritis and possibly other chronic inflammatory diseases.

NF-κB may also have a prominent role in rheumatoid arthritis joint destruction as it promotes osteoclast maturation and increased bone-resorbing activity (Iotsova et al., 1997), and inhibits at the same chondrocyte differentiation and repair of damaged cartilage tissue (de Crombrugghe et al., 2000; Murakami et al., 2000). In animal models of arthritis, the genetic inactivation of c-Rel and NF-κB1 in $rel^{-/-}$- and $nfkb1^{-/-}$-deficient mice protected the animals from the development of collagen-induced arthritis (CIA) (Campbell et al., 2000), as did the

transgenic expression of (superrepressor) IκBα in cells of the T-cell lineage (Seetharaman et al., 1999). Similarly, the administration of NF-κB decoy oligonucleotides suppressed the severity of already established disease in both CIA and SCW rat arthritis by inhibiting the production of TNFα and IL-1 within the joints and by reducing paw swelling and disease activity (Miagkov et al., 1998; Tomita et al., 1999). A proteosome inhibitor that prevents IκB degradation also afforded protection in SCW-induced rat arthritis (Palombella et al., 1998). Thus, blocking NF-κB may reduce the inflammatory response and restore the cytokine equilibrium in the rheumatoid joint by reducing at the same time cartilage and bone damage.

3.3. Inflammatory bowel disease (IBD)

IBD comprises a heterogeneous group of diseases including Crohn's disease (CD) and ulcerative colitis (UC). Several genetic and environmental factors are implicated in the pathophysiology of IBD that ultimately result in a common final manifestation, which is mucosal inflammation (Fiocchi, 1998). Deregulated cytokine production and signaling mechanisms by epithelial cells, mucosal lymphocytes and macrophages seem to play a major role in the inflammatory response that takes place in the colonic mucosa. NF-κB, in particular, has been found to be increased in patients with IBD and p65 to be localized in epithelial cells and lamina propria macrophages in biopsies of patients (Neurath et al., 1996; Neurath & Pettersson, 1997; Rogler et al., 1998; Schreiber et al., 1998). Increased NF-κB DNA-binding activity has been demonstrated in nuclear extracts from both biopsy specimens and isolated lamina propria mononuclear cells from CD patients (Schreiber et al., 1998), and in vitro treatment of lamina propria macrophages with p65 antisense oligonucleotides was shown to be more effective in downregulating the expression of TNFα, IL-1β and IL-6 than 5-aminosalicylic acid or glucocorticoids (Neurath et al., 1996; Neurath & Pettersson, 1997). Collectively, these observations suggested that NF-κB activation may be important in the generation and maintenance of mucosal inflammation, although its activation may not be specific to IBD. Indeed, increased NF-κB levels have also been found in nonspecific colitis or diverticulitis biopsy speciments and were not significantly different from those found in CD or UC specimens (Rogler et al., 1998).

NF-κB was also found to be activated in several animal models that closely resemble human IBD. Thus, in TNBS-induced colitis in mice, increased NF-κB DNA-binding activity, that consisted mainly of the p65 and p50 NF-κB subunits, was detected in nuclear extracts from lamina propria macrophages (Neurath et al., 1996). Inhibition of this activity in vivo by intravenous or local colonic administration of p65 antisense oligonucleotides abrogated the clinical signs of established colitis (Neurath et al., 1996; Neurath & Pettersson, 1997). Strikingly, the efficacy of p65 antisense oligonucleotides in treating TNBS-induced colitis appeared to be superior than the administration (single or daily) of glucocorticoids. In addition, in IL-10-deficient mice, another model of chronic intestinal

inflammation (Kuhn et al., 1993), increased levels of NF-κB DNA-binding activity and increased levels of p65 protein expression were found in lamina propria macrophages. Again, administration of p65 antisense oligonucleotides improved the clinical and histological signs of established colitis (Neurath et al., 1996). Finally, in DSS-induced colitis in mice, intravenous or oral administration of an inhibitor that blocks NF-κB nuclear translocation, reduced both the inflammation and injury scores of the diseased colon (Fujihara et al., 2000). These in vivo studies suggest that specific blockade of NF-κB may also have therapeutic efficacy in the human forms of colonic intestinal inflammation such as CD and UC.

3.4. Psoriasis

Psoriasis is a chronic remitting-relapsing skin disease. It is caused by increased epidermal proliferation and infiltration of inflammatory/immune cells into the epidermis that result in raised scaly plaques on the skin surface and skin lesions. This condition is often seen in conjunction with nail disease and arthritis. Numerous inflammatory mediators have been reported to be upregulated in skin biopsy speciments from psoriatic lesions, including TNFα, IL-1 and IL-15 (Danning et al., 2000). The p65 NF-κB subunit has also been shown to be activated in psoriatic lesions, although it is not known whether this is a cause or consequence of the inflammatory process taking place (Danning et al., 2000). Certainly, experiments in an animal model of psoriasis using a selective proteasome inhibitor that prevents IκB degradation reduced the severity of psoriasis, indicating that NF-κB activation may indeed be essential in that process (Zollner et al., 2002). In addition, the observation that cyclosporin, glucocorticoids and fumaric acid esters, commonly used for the treatment of psoriasis in humans (Asadullah et al., 2002), all can inhibit NF-κB activation, also supports this possibility.

3.5. Inflammatory lung diseases

Acute and chronic alveolar and/or bronchial inflammation also takes place in a number of diseases of the lung, collectively termed inflammatory lung diseases. These include acute lung injury (ALI), acute respiratory distress syndrome (ARDS), fibrosing alveolitis (FA) and asthma, and are characterized by an inflammatory response that involves the recruitment to the lungs and the activation of immune cells. This is accompanied by the upregulation of adhesion molecules and the secretion of cytokines, chemokines, oxidants and other inflammatory mediators, and results in lung injury. Airway epithelial cells contribute to that process as they are also able to produce inflammatory mediators (Thompson et al., 1995a).

NF-κB seems to be important in lung inflammation as increased NF-κB activity has been detected in bronchoalveolar lavage biopsies from ALI (Blackwell & Christman, 1997), ARDS (Schwartz et al., 1996; Christman et al., 1998), FA

(Conron et al., 2002) and asthma patients (Hart et al., 1998) compared to healthy volunteers (Farver et al., 1998). In asthma patients in particular, increased NF-κB activity is also present in the submucosal cells, in the endothelium and the bronchial epithelium (Barnes & Adcock, 1997). Few studies have been performed to evaluate the role of NF-κB in lung inflammation. In bronchoalveolar lavage biopsies from FA patients, we recently showed that overexpression of IκBα inhibits the constitutive production of both macrophage and T-cell-derived cytokines such as TNFα, IL-6, IL-8 and IFNγ, demonstrating that NF-κB regulates the expression of a number of inflammatory mediators in this system (Conron et al., 2001). The examination of putative kinases that may be involved in that process revealed that IKK2, but not NIK was required (Conron et al., 2002). Several animal models of lung inflammation have also been used. In rat and mouse models of endotoxic shock, intraperitoneal administration of endotoxin induces NF-κB activation in alveolar macrophages and lung tissue that is associated with lung neutrophilia, epithelial permeability and lipid peroxidation (Blackwell et al., 1996; Blackwell et al., 1999). This was suppressed by treatment with antioxidants that inhibit NF-κB activation (Blackwell et al., 1996). Similarly, hemorrhage-induced shock in mice resulted in rapid NF-κB activation in intraparenchymal lung mononuclear cells and in lung inflammation (Shenkar & Abraham, 1997). This may occur through stimulation of α-adrenergic receptors and the generation of reactive oxygen species. Finally, mice deficient in the p50 NF-κB subunit of NF-κB are incapable of mounting eosinophilic airway inflammation. This is due to the lack of production of IL-5 and eotaxin, MIP-1α and MIP-1β, but not to T-cell activation or adhesion molecule expression (Yang et al., 1998). The same was true for c-Rel-deficient mice that were resistant to allergen-induced pulmonary inflammation, airway hyperresponsiveness and eosinophilia (Donovan et al., 1999). As these are common features of asthma, it is likely that p50, c-rel and probably other NF-κB subunits are also important in the human disease. Although the stimuli for NF-κB activation in asthmatic lungs have not been defined, agents that correlate with asthma exacerbations, such as various allergens, ozone, rhinoviruses and respiratory syncytial viruses, have all been shown to activate NF-κB (Christman et al., 2000).

3.6. Atherosclerosis

Atherosclerosis and its clinical manifestations of heart attack, stroke and peripheral vascular insufficiency can be viewed as a multistep, chronic inflammatory disease. The development of atherosclerotic lesions can be subdivided into three phases, viz. the initiation phase (formation of small fatty streaks), the expansion phase (endothelial cell growth and coalescence of fatty streaks) and the progression phase to plaques (recruitment of smooth muscle cells, deposition of collagen and formation of a fibrous cap). This process is under the control of cytokines, chemokines, growth factors and other soluble mediators and adhesion molecules (produced within the lesion) that regulate the recruitment and proliferation of

monocytes and macrophages to the vessel wall, the migration of smooth muscle cells from the media to the intima of the vessel, and the proliferation of vascular endothelial cells. As many of these inflammatory mediators can be regulated by NF-κB, it has been suggested that NF-κB may also play a major role in the pathogenesis of atherosclerosis (Collins & Cybulsky, 2001). Indeed, activated NF-κB has been detected in human atherosclerotic plaques, whereas it is almost absent in healthy vessels devoid of atherosclerosis. Studies using an antibody that recognizes p65 dissociated from IκB revealed that activated p65 localizes in the intima and media of atheromatous areas of atherosclerotic lesions in smooth muscle cells, macrophages and endothelial cells (Brand et al., 1996). This is in contrast to healthy vessels where p65 and p50 are mainly localized in the cytoplasm. Elevated NF-κB levels have also been detected in arterial smooth muscle cells after injury and have been suggested to account for the expression of several genes, including ICAM-1, VCAM-1 and MCP-1, all of which facilitate the recruitment of monocytes and macrophages to the atherosclerotic lesions (Landry et al., 1997).

Few studies have also examined the activation of NF-κB in animal models of atherosclerosis. One study demonstrated activated NF-κB in intimal cells from coronary arteries of pigs fed with a hypercholesterolemic diet (Wilson et al., 2000). Interestingly, another study in mice suggested that there is elevated NF-κB expression in vessel regions of high probability for the development of atherosclerotic plaques when compared to adjacent regions where plaques are unlikely to develop (Hajra et al., 2000). As these high probability regions coincide with areas exposed to high shear stress, the authors of the study proposed that shear stress may prime certain regions of the vessel wall by inducing steady state levels of NF-κB that may upregulate the expression of cytokines and adhesion molecules upon an additional atherogenic stimulus. In humans, many atherogenic stimuli or risk factors have been shown to activate NF-κB. Thus, oxidative stress (Li & Karin, 1999), oxidized LDL and VLDL (Brand et al., 1997; Calara et al., 1998; Dichtl et al., 1999), hypertension and angiotensin II (Han et al., 1999; Pueyo et al., 2000), hyperglycemia and elevated advanced glycation end products (Morigi et al., 1998; Schmidt et al., 1999), homocysteine and various infectious agents such as cytomegalovirus and Chlamydia pneumonia (Welch & Loscalzo, 1998) have all been shown to induce NF-κB activation in vitro or in vivo. In summary, there is good evidence that deregulated NF-κB may be involved in the development, but also some of the pathological features of atherosclerosis, although more studies are needed to demonstrate that blockade of NF-κB can result in therapeutic benefit in animal models of the disease and ultimately patients.

3.7. Diabetes

There are two forms of diabetes, viz. type 1 (often referred to as insulin-dependent diabetes mellitus or juvenile diabetes) and type 2, known as insulin-independent diabetes or adult onset diabetes. Type 1 is the most serious and is caused by

the autoimmune apoptotic destruction of the insulin-producing beta cells in the islets of Langerhans. This involves the activation of T-cells and antigen-presenting cells, and the production of cytokines such as INFγ, TNFα and IL-1β (Igaz et al., 2000). Type 2 diabetes develops slowly and is probably due to alterations in fat metabolism as the body gains weight and becomes resistant to insulin. Common complications of diabetes include hypertension and cardiovascular disease.

Inappropriate NF-κB activation has recently been implicated in both types of diabetes. In type 1 diabetes, peripheral blood mononuclear cells from patients have increased NF-κB DNA-binding activity (Hofmann et al., 1998; Bierhaus et al., 2001) and increased NF-κB DNA-binding activity has been associated with more pronounced diabetic complications such as diabetic nephropathy (Hofmann et al., 1999; Mohamed et al., 1999). In human islets, the expression of a mutated nondegradable form of the IκBα or the antiapoptotic gene A20 (that also inhibits NF-κB in this system) prevents cytokine-induced beta cell death (Grey et al., 1999; Giannoukakis et al., 2000; Heimberg et al., 2001) by blocking the apoptotic pathway and probably by preventing the NF-κB-dependent upregulation of the Fas gene (Darville & Eizirik, 2001). Evidence for an important role of NF-κB in type 1 diabetes also comes from studies in animal models. In chemically induced diabetes (alloxan and steptozotocin-induced diabetes) in mice, administration of NF-κB decoy oligonucleotides (Quan et al., 2001) or other NF-κB inhibitors such as dietary zinc and Amomum xanthoides extract (Ho et al., 2001; Park & Park, 2001) inhibited the development of diabetes. In addition, nonobese diabetic (NOD) mice that are commonly used to model human type 1 diabetes exhibit elevated levels of NF-κB activation that leads to enhanced DC and T-cell function and increased autoimmune destruction of the islets of Langerhans (Hayashi et al., 1993; Weaver et al., 2001; Poligone et al., 2002). Polymorphisms in the NF-κB locus have recently been associated with human type 1 diabetes (Hegazy et al., 2001).

NF-κB has also been implicated in the development of insulin-resistance that leads to type II diabetes. The discovery that salicylates used to treat insulin-resistance potently inhibit IKKβ (Yin et al., 1998) and the observation that IKKβ-deficient mice are protected from the development of fat-induced insulin resistance (Kim et al., 2001) suggest that NF-κB activation may be essential for that process.

3.8. Neurodegenerative diseases

3.8.1. Scope

Most cells within the nervous system (including neurons, astrocytes, microglia and oligodendrocytes) express functional NF-κB (Kaltschmidt et al., 1994; O'Neill & Kaltschmidt, 1997). This can be upregulated by a wide spectrum of stimuli that include reactive oxygen species (Schreck et al., 1992), inflammatory cytokines (Barger et al., 1995), viral and bacterial products, depolarization

(Guerrini et al., 1995; Kaltschmidt et al., 1995), nerve growth factor (Carter et al., 1996), β-amyloid (Barger & Mattson, 1996; the secreted form of β-amyloid precursor protein) and dopamine (Weingarten et al., 2001). Recently, NF-κB activation was suggested to play an important role in the development of many neurodegenerative diseases by regulating survival and growth of neurons, microglia and astrocytes.

3.8.2. Multiple sclerosis (MS)

MS is a demyelinating disease where myelin in the central nervous system is lost. This is a result of an immune response that develops against oligodendrocytes (the cells that produce myelin) and effectively inhibits their function and induces their depletion (Kieseier et al., 1999). The mechanisms, however, that regulate these events remain poorly understood. Studies by immunohistochemistry have shown that NF-κB is upregulated and localized in the nuclei of microglia and oligodendrocytes in chronic active MS lesions (Gveric et al., 1998; Bonetti et al., 1999). As IL-1 and TNFα are also localized in active MS lesions, these cytokines are likely to account for the observed upregulation of NF-κB (Hofman et al., 1989; Selmaj et al., 1991; Woodroofe & Cuzner, 1993; Liu et al., 1996; Bierhaus et al., 2001). Upregulated NF-κB, in turn, may amplify the inflammatory process taking place in MS by increasing the expression of NF-κB-controlled adhesion molecules and cytokines. Ultimately, this leads to disease progression, disability and even death.

In experimental encephalomyelitis (EAE), an animal model of MS and autoimmune neural damage, NF-κB activation has also been reported (Pahan & Schmid, 2000). Interestingly, NF-κB-deficient mice are resistant to induced EAE, possibly due to a deficiency in the differentiation of myelin oligodendrocyte glycoprotein-specific T-cells to Th1- or Th2-type effector cells (Hilliard et al., 1999). However, although inflammatory factors that become upregulated by NF-κB may act to promote autoimmune disease, NF-κB may at the same time upregulate many antiapoptotic genes, thus protecting neurons and oligodendrocytes from cell death and further damage (Beg & Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996; Bonetti et al., 1997; Bonetti & Raine, 1997). The pathogenesis of this disease may thus rely on the balance between the 'beneficial' and 'pathological' effects of NF-κB.

3.8.3. Alzheimer's disease (AD)

AD is a neurodegenerative disease that affects the elderly. The main symptoms are memory loss and dementia, with the severity of disease being linked to the amplitude of neuronal and synaptic loss. Aggregates of amyloid beta peptides build up forming plaques along with neurofibrilary tangles in neurons that interfere with the normal function of the brain (Walsh et al., 2002). Interestingly, the areas of the brain that are affected by AD pathology also demonstrate increased NF-κB activity (Yan et al., 1995). Thus, p65 NF-κB has been localized in neurons and astrocytes next to amyloid plaques found in brain sections from AD

patients (Terai et al., 1996; Kaltschmidt et al., 1997; O'Neill & Kaltschmidt, 1997; Ferrer et al., 1998). A mechanism by which NF-κB may worsen AD has been proposed. NF-κB can be activated by a number of stimuli that include soluble amyloid precursor protein (Barger & Mattson, 1996), β-amyloid peptides possibly through activation of RAGE (Kaltschmidt et al., 1997), IL-1β and glutamate (Grilli et al., 1996a). This creates a positive regulatory loop as NF-κB also upregulates the expression of the amyloid precursor protein gene (Grilli et al., 1996a), producing higher levels of β-amyloid peptide that further activates NF-κB and induces neuronal cell death. The observation, however, that NF-κB can also be beneficial in AD by upregulating the expression of genes like SOD (Mattson et al., 1997; Keller et al., 1998) and other antiapoptotic proteins that may aid neuronal survival (Barger et al., 1995; Zong et al., 1999) has complicated matters. Therefore, it is still unclear whether NF-κB is a potential therapeutic target in AD, and more studies are needed.

4. NF-κB IN THERAPY

Although there is a large amount of data that deregulated NF-κB is central to the pathogenesis of a number of inflammatory diseases, the therapeutic potential of NF-κB blockade has not yet been tested in the clinic. Evidence, however, from other immunosuppressive and anti-inflammatory drugs already used for the treatment of these diseases (Table 3) suggests that it is likely to be very beneficial, as the common property of these drugs is their ability to inhibit NF-κB activation among their multiple effects.

Table 3. Drugs used for the treatment of inflammatory diseases that have been proposed to block NF-κB.

Disease	*Treatment*
Rheumatoid arthritis	Glucocorticoids, NSAIDs, cyclosporin A and tacromilus, methotrexate, sulfasalazine, leflunomide, gold and D-penicillamine compounds, anti-TNFα blocking agents
IBD	Glucocorticoids, NSAIDs, cyclosporin A and tacromilus, methotrexate, sulfasalazine, anti-TNFα blocking agents
Psoriasis	Glucocorticoids, NSAIDs, cyclosporin A and tacromilus, methotrexate, anti-TNF blocking agents
Inflammatory lung diseases	Glucocorticoids, NSAIDs, cyclosporin A and tacromilus
Atherosclerosis	NSAIDs
Insulin resistance (diabetes)	NSAIDs
Neurodegenerative diseases	Glucocorticoids, NSAIDs, cyclosporin A and tacromilus

Thus glucocorticoids, such as dexamethasone and prednisone, which are frequently used for the treatment of rheumatoid arthritis, IBD and inflammatory lung diseases (Geier & Miner, 1992; Caesar et al., 1997; Keatings et al., 1997; Norbiato et al., 1997; Wilckens & De Rijk, 1997) owe, at least in part, their

therapeutic effects to their ability to inhibit NF-κB-dependent gene expression. Glucocorticoids have been shown to upregulate the transcription and expression of IκBα, possibly by yet unidentified glucocorticoid-responsive elements in the IκBα promoter (Auphan et al., 1995; Scheinman et al., 1995a), and to physically interact with p65, thereby preventing transactivation of NF-κB (Caldenhoven et al., 1995; Scheinman et al., 1995b; De Bosscher et al., 1997; Heck et al., 1997).

In addition, NSAIDs were recently suggested to inhibit NF-κB activity, an effect that could account for their anti-inflammatory action. Thus, sodium salicylates and aspirin were shown to inhibit IκBα phosphorylation and degradation by preventing ATP-binding to IKK2 at concentrations found in the serum of patients treated for inflammatory conditions (Kopp & Ghosh, 1994; Grilli et al., 1996b; Pierce et al., 1996; Yin et al., 1998). At the same time, methotrexate, sulfasalazine and leflunomide were shown to inhibit IκBα phosphorylation and degradation, probably by interfering with the IKK complex (Egan & Sandborn, 1998; Wahl et al., 1998; Manna et al., 2000; Majumdar & Aggarwal, 2001), and demonstrating that certain NSAIDs may exert their anti-inflammatory effects by blocking the activation of NF-κB.

Similarly, gold and D-penicillamine compounds and thalidomine, that are used drugs, especially for the treatment of rheumatoid arthritis, all have inhibitory effects on NF-κB activation (Yang et al., 1995; Keifer et al., 2001). Immunosuppressive agents such as cyclosporin A and tacrolimus (FK-506), that are mainly employed in transplantation, but also in inflammatory diseases such as rheumatoid arthritis, were also able to block the activation of NF-κB in addition to their effects in other pathways such as the activation of NF-AT. Cyclosporin A has been claimed to prevent IκBα degradation and NF-κB activation by interfering with the proteasome function (Marienfeld et al., 1997; Meyer et al., 1997), whereas tacromilus seems to specifically inhibit c-Rel, but not RelB or p50 nuclear translocation (Venkataraman et al., 1995). Finally, anticytokine biologicals such as anti-TNFα blocking agents, shown to be highly successful in the treatment of a number of inflammatory diseases (Andreakos et al., 2002), may owe part of their efficacy to the prevention of cytokine-induced NF-κB activation and NF-κB-dependent gene expression. Support for that comes from a study in refractory Crohn's disease, where anti-TNFα blocking agents have been demonstrated to induce disease remission in 30-50% of the patients. It was shown that the anti-TNFα blocking agent infliximab reduced significantly nuclear p65 expression in the colonic mucosa within one week after a single infusion, and an extended downregulation of p65 seemed to be associated with a sustained induction of remission (Nikolaus et al., 2000). In contrast, reaccumulation of nuclear p65 correlated with the increase in TNFα secretion, and was usually observed before patients presented with a clinical relapse. These findings indicated that the activation of NF-κB may be secondary to the secretion of TNFα, and suggested a possible mechanism by which infliximab improves Crohn's disease.

However, as the majority of these drugs is associated with various side effects, especially in the long term, the specific inhibition of NF-κB may result in

increased therapeutic efficacy and reduced toxicity. Initial attempts to identify novel NF-κB inhibitors focused on interfering with proteasome function or using natural plant products such as flavonoids to prevent IκB degradation and NF-κB activation. However, it became apparent that these inhibitors would also lack specificity and thus be subject to more severe side effects. Current attempts are focusing on the design of specific NF-κB inhibitors that block NF-κB without affecting other pathways. This requires the identification of upstream signaling components that lead to the activation of NF-κB, the determination of which of these are activated and rate-limiting in disease states, as well as the elucidation of their molecular structure and biochemical properties. In that way, some of the main worries concerning NF-κB-specific drugs, such as liver toxicity or interference with host defences in the long term (Beg et al., 1995; Li et al., 1999a-b; Tanaka et al., 1999; Lavon et al., 2000; Rudolph et al., 2000) may be overcome. Certainly, the fact that NSAIDs or glucocorticoids, that block NF-κB, can be tolerated long-term indicates that the use of NF-κB inhibitors is a valid strategy. Of course, under conditions where NF-κB inhibitors will be needed for short-term therapy, these worries are not of much concern. Ultimately, clinical trials will be needed to resolve these issues and assess the potential of NF-κB inhibitors as novel anti-inflammatory agents as well as their therapeutic efficacy.

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