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Commentary

Anti-inflammatory interventions of NF-κB signaling: Potential applications and risks

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ABSTRACT

Signaling via NF-κB is a key process during inflammation and thus constitutes an attractive target for anti-inflammatory therapeutic interventions. Especially during initial hyperinflammatory states of an acute illness such as sepsis or in the course of chronic inflammation and autoimmune diseases inhibition of IKK-driven NF-κB activation provides a promising treatment strategy. Given its critical role in innate and adaptive immune responses, however, there is a certain amount of risk due to induced immunodeficiency that may follow inhibitory treatment. Moreover, its primary anti-apoptotic function suggests that blockade of NF-κB activation has dramatic effects on cell functions and survival and eventually worsens the course of an inflammatory disease.

An overview of canonical and alternative NF-κB activation and its critical role in immune responses will be provided. A main topic focuses on recent animal studies and data derived from genetic studies in humans that provide an insight into potential effects of different therapeutic modulations of NF-κB inflammatory signaling. The pros and cons of NF-κB inhibition and treatment strategies will be critically reviewed.

1. Introduction

Since the discovery of the inducible transcription factor NF-κB more than 20 years ago by Sen and Baltimore [1] an overwhelming amount of information concerning its function and regulation has accumulated. Thus, NF-κB signaling has become a main topic in the field of immunology and cancer biology. Initially, most of our current knowledge of NF-κB derived from investigations concerning the induction and regulation of the immune response. However, the role of NF-κB in tumorigenesis and cancer therapy becomes increasingly evident. In keeping with this fact numerous reviews have been published recently covering the impact of NF-κB activation on cell life and death decisions, tumor promotion and suppression, the linkage of inflammation to cancer, hematologic malignancies and cancer therapy [2–4]. This review, therefore, will focus on diverse functions of NF-κB in the context of inflammation and immune answers. By illuminating those mechanisms of NF-κB signaling that are potentially relevant for treatment strategies different indications of NF-κB modulation in the context of inflammatory disorders will be discussed.

2. Overview of molecular NF-κB signaling

First described in B cells, NF-κB has soon been shown to exist in an inactive cytosolic form in all cell types. The term NF-κB

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stands for a family of five structurally related and evolutionarily conserved mammalian proteins (also known as rel proteins): RelA (p65), RelB, c-Rel (REL), NF-κB$_1$ (p50 and its precursor p105), and NF-κB$_2$ (p52 and its precursor p100). The proteins share an N-terminal Rel homology domain (RHD) of about 300 amino acids that is responsible for dimerization, interaction with inhibitory proteins of NF-κB (IκBs), nuclear translocation, and DNA binding [5]. In resting cells, NF-κB dimers are retained in the cytoplasm by interaction with inhibitory proteins of the IκB family. RelA, RelB, and c-Rel contain C-terminal transactivation domains (TAD) and are expressed as mature proteins. In contrast, NF-κB$_1$ (p50) and NF-κB$_2$ (p52) result from proteolytic processing of the precursor proteins p105 and p100, respectively. Both proteins lack transactivation domains and thus p50 and p52 homodimers have been shown to repress transcription in transfection studies. Association with appropriate co-activators such as CREB-binding protein, however, may positively regulate transcription by these homodimers [6]. The precursor proteins p100 and p105 contain C-terminal IκB-typical ankyrin repeats and thus also retain bound Rel proteins in the cytosol [5]. The IκB family consists of three subgroups: the classical IκBs (IκBα, IκBβ, IκBγ), the NF-κB precursors (p100, p105), and the unusual IκBs (Bcl-3, IκBξ, IκB$_{NS}$). The IκBs function by masking the nuclear localization sequence (NLS) in the Rel homology domain in one of the subunits of the p50/p65 heterodimer which allows the NF-κB/IκB complex to cycle in and out of the nucleus. IκBα is further known to block DNA binding [5].

The NF-κB pathway is induced by a large number of extracellular signals involved in both innate and adaptive immune answers and in regulation of apoptosis. Upon activation of different key regulatory receptors such as Toll-like receptors responsible of the recognition of pathogenic microorganisms [7], IL-1 and TNF receptor family members (including death receptors), and B- and T-cell receptor [8,9] the IκB proteins are phosphorylated by the inhibitor of κB (IκB) kinase complex (IKK). IKK consists of two catalytically active subunits, IKKα (IKK1) and IKKβ (IKK2), and the regulatory and structural subunit IKKγ (also known as NF-κB essential modulator—NEMO).

NF-κB signaling occurs through the classical (canonical) or the alternative (non-canonical) pathway [10,11] (Fig. 1). During classical activation the IKK complex specifically phosphorylates IκBs on two conserved N-terminal serine residues which targets them for E2- and E3-ligase-mediated polyubiquitination and subsequent 26S proteasomal degradation. This process releases and thus activates NF-κB which now translocates to the nucleus and regulates the transcription of response genes encoding chemokines, cytokines, adhesion molecules, inflammation-associated enzymes and inhibitors to apoptosis [5,12]. The classical NF-κB pathway has been closely linked to the orchestration of inflammatory responses and survival of professional immune cells by coordinated expression of multiple inflammatory and innate immune genes.

Whereas classical NF-κB activation is IKKβ and Ikkγ-dependent and occurs through IκBα degradation, the alternative pathway depends on IKKα homodimers and NF-κB-inducing kinase (NIK) and results in regulated processing of the p100 precursor protein to p52 via phosphorylation and degradation of its IκB-terminus [10,11,13] (Fig. 1). Most commonly associated with RelB, activation of this pathway leads to nuclear translocation of p52-RelB heterodimers [13]. This alternative pathway is primarily stimulated by ligation of specific TNF superfamily receptors, e.g. by lymphotoxinβ (LTβ), B-cell-activating factor (BAFF), and CD40L [14], RANK, CD27, CD30 and is particularly important for B-cell maturation and lymphoid organ formation [10,11,13].

However, recent data provide evidence that classical and alternative NF-κB signaling may not dogmatically be separated. Both, IKKα and IKKβ are able to phosphorylate IκB in vitro, but IKKβ demonstrates higher kinase activity than IKKα [5]. Certain stimuli such as RANKL and LTβ signal to both the canonical and the alternative pathway [11]. Moreover, Luedde and co-workers [15] have shown in hepatocytes that IKKβ deficiency has no effect on TNFα-induced NF-κB activation. In contrast NEMO-deficient hepatocytes do not activate NF-κB upon TNFα stimulation. These data suggest that a remaining NEMO/IKKα complex is sufficient to fully activate NF-κB in the absence of IKKβ. However, it cannot be ruled out that the catalytically active subunits of an intact IKK complex fulfill separated tasks to some extent. This might occur via recruitment of subunits. Thus, under physiological conditions the IKK complex is likely to direct, at least to some extent, the signal to the canonical and alternative NF-κB pathway depending on the type of stimulus. Moreover, recent studies suggest a role of IKKα in the classical pathway as it may regulate gene expression in the nucleus by modifying the phosphorylation status of histones [8].

Regarding the exact knowledge of mechanisms involved in NF-κB activation its negative regulation is still poorly understood. Activated NF-κB is downregulated through multiple mechanisms including the feedback loop of de novo IκBα gene expression by NF-κB, which is likely to play a key role. IκBα binds to nuclear NF-κB and exports it to the cytosol thus terminating its activity [5]. Posttranslational modifications such as phosphorylation and acetylation of RelA-containing complexes are known to positively correlate with duration of the NF-κB response [16]. The association of RelA with histone deacetylase (HDAC) corepressor proteins, however, suggests that the repression of NF-κB-regulated genes is due to increased deacetylation of RelA. Consequently, it was shown that Inhibition of HDAC activity increases both basal and TNF-induced expression of the NF-κB-regulated interleukin-8 (IL-8) gene [17]. This may be explained in part by an increased binding affinity of IκBα to deacetylated RelA [16]. Taken together, increased deacetylation of RelA and histones promotes the shut down of the NF-κB response. These posttranslational modifications of NF-κB-mediated cellular responses are currently the focus of intense research and might be a target of future therapeutic modulations.

3. NF-κB and the immune response

3.1. *Impact on differentiation and basic cellular functions in innate immune cells*

NF-κB has been shown to be involved in the differentiation and activation of macrophages, osteoclasts, DCs and granulocytes

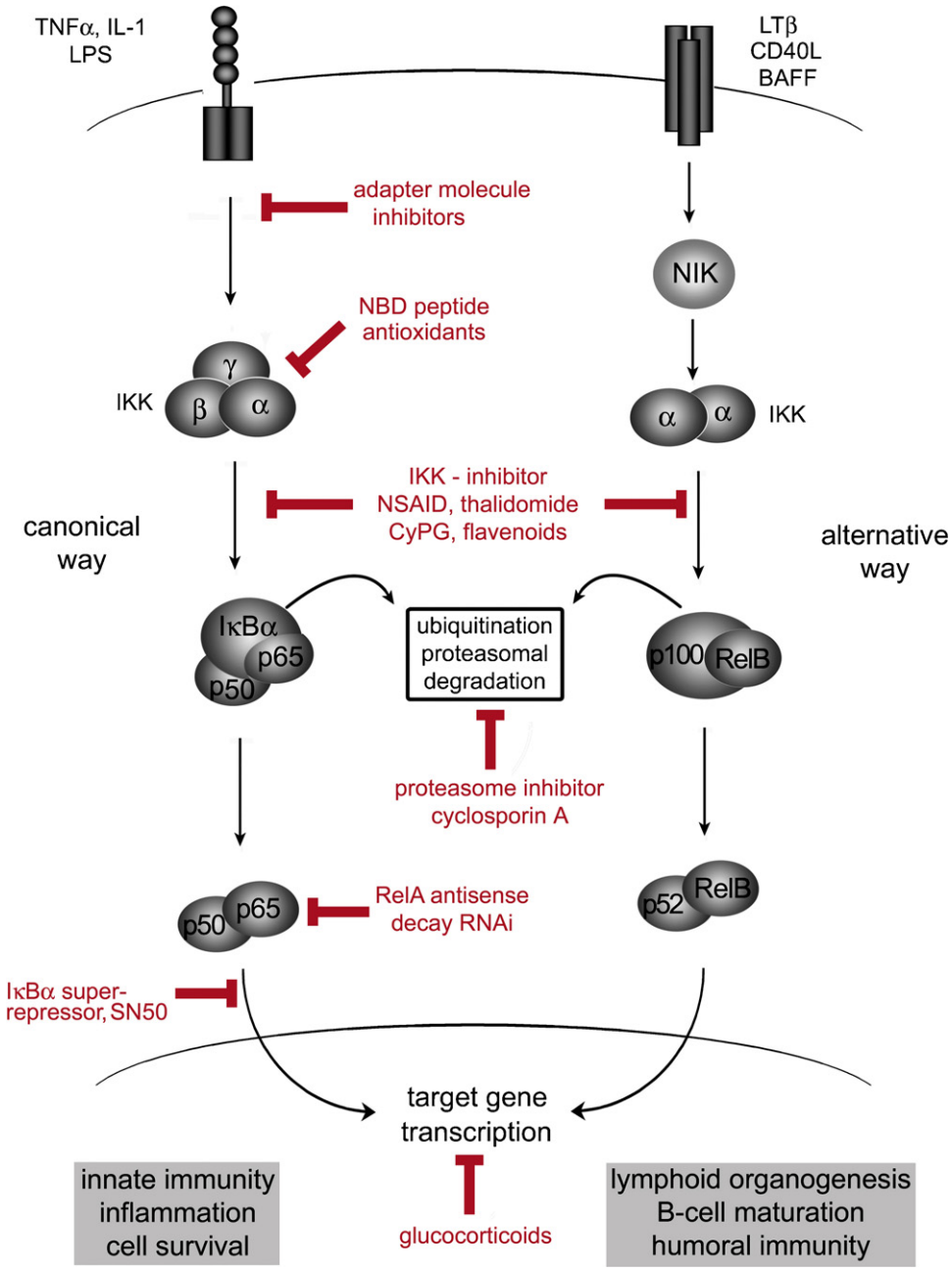

Fig. 1 – Schematic representation of both, the canonical and the alternative NF-κB signaling pathway and inhibitory tools of NF-κB activation (see text for details, modified according to [75,76]).

[9]. Monocyte precursors do not exhibit NF-κB activity whereas their differentiation into macrophages involves NF-κB-dependent transcription of anti-apoptotic target genes [18]. In macrophages, classical NF-κB signaling is induced, e.g. upon engagement of microbial molecules with Toll-like receptors (TLRs) and thus is particularly important in establishing innate immunity. Moreover, classical NF-κB signaling in macrophages essentially contributes to antigen processing/presentation and co-stimulation of lymphocytes [19], a fact which underscores this pathway to be a key player in the orchestration of innate and adaptive immune answers. The key enzyme of the alternative pathway, IKKα, however, was recently shown to negatively control classical NF-κB signaling in macrophages. Lawrence et al. [20] have shown that inactivation of IKKα in mice enhances LPS-induced inflammation and bacterial clearance in vivo. Via modulation of p65 phosphorylation IKKα leads to an accelerated turnover of the transcription factor in macrophages which ultimately promotes the resolution of inflammation [20].

NF-κB signaling also plays a prominent role in the differentiation process from monocyte/macrophage precursors to osteoclasts. It was shown that ligation of RANK (receptor activator of NF-κB) on osteoclasts by RANK ligand (RANKL) on osteoblasts leads to activation of osteoclasts via

TRAF6-mediated NF-κB signaling [21]. Disruption of both the *rank* and *rankl* gene results in severe osteopetrosis in mice [22,23]. Also, mice deficient for both p50 and p52 as well as TRAF6 exhibit an osteopetrosis phenotype [24,25]. The different roles addressed to both the classical and the alternative NF-κB pathway on osteoclastogenesis, however, are not entirely clear. Hence, no osteopetrotic phenotype has been reported in NIK-deficient or mutant aly/aly (point mutation in the *nik* gene that prevents NIK activation) mice although NIK-deficient bone marrow cells are unable to differentiate into mature osteoclasts upon RANK stimulation [26]. Indeed, NIK/IKKα and thus the alternative way seems not to be involved in basal osteoclastogenesis. Although IKKα is required for RANK ligand-induced p100 processing and osteoclast formation in vitro, mice with an inducible deletion of IKKβ ($IKK\beta^{\Delta}$) in hematopoietic cells demonstrated that only IKKβ and, hence, the classical NF-κB activation pathway is necessary for osteoclastogenesis and anti-apoptosis of osteoclasts in vivo [21].

Dendritic cells (DCs) represent a key type of antigen-presenting cell (APC) and are important for the development of innate and adaptive immunity. DCs are involved in T-cell priming and activation via processing/presentation of foreign antigens. RelB and p52 are specifically expressed in DCs suggesting a prominent role of the alternative pathway in these cells. RelB was shown to facilitate the development of $CD8^-$ DCs [9] and is required for the processing and presentation of antigens by residual DCs [27]. Models to study the canonical NF-κB pathway in DCs, such as p50-RelA double-knockout studies, however, have demonstrated complete absence of both $CD8^+$ and $CD8^-$ DCs and no defect of other myeloid cells could be detected [9]. Moreover, overexpression of a non-degradable IκBα super-repressor inhibits the maturation of DCs and results in significantly decreased survival [28]. This is in line with data derived from blocking NF-κB activity in DC by selectively targeting the IKK complex with the NF-κB inhibitor NEMO-binding domain (NBD) peptide. This approach showed strongly reduced DC maturation and marked inhibition of T-cell proliferation in an allogeneic mixed lymphocyte reaction which was accompanied by less T_H1 and T_H2 polarization [29]. Taken together, these results point to the pivotal role of both canonical and alternative NF-κB pathways for DC maturation, function and survival.

During granulocyte differentiation NF-κB is thought to be constitutively activated via an autocrine loop induced by chemokines/cytokines and their respective receptors which finally leads to transition of metamyelocytes to bone marrow PMNs [30]. IκBα knockout mice display granulocytosis [31] suggesting an anti-apoptotic role during granulocyte development. Mature normal granulocytes, however, primarily express NF-κB-dependent anti-apoptotic genes upon stimulation of various TLRs [32]. They also lack p52 and RelB [33] that are characteristic for long-lived lymphocytes [34]. Thus, primarily the canonical NF-κB pathway promotes basic innate immune cell functions such as migration [35]. Taken together, the main function of NF-κB in PMNs aims to act as prosurvival and proinflammatory factor which is particularly important during the inflammatory response and the defense of invading microbes.

Myeloid cells are key players during innate immune answers and promote the onset of an adequate adaptive immune response. As a first step, innate immunity involves the recognition of specific microbial signature motifs. LPS, dsRNA or bacterial flagellin are examples of those microbial molecules that contain so called pathogen-associated molecular pattern (PAMP). PAMPs are recognized by pathogen recognition receptors (PRRs). PRRs include Toll-like receptors (TLRs), cytoplasmic receptors (nucleotide oligomerization domain proteins, NOD), scavenger receptors and the complement system. Microbial activation of NF-κB via TLRs is a key mechanism of innate immunity and is increasingly understood [7]. TLRs contain extracellular leucine-rich repeats responsible for the recognition of PAMPs, and transmembrane and cytoplasmic Toll/interleukin-1 receptor (TIR) domains that are required for intracellular signaling [7]. Proximal steps of TLR signaling can be separated into two pathways that depend on two adapter proteins: MyD88 (myeloid differentiation primary response protein 88) and Trif (TIR domain-containing adapter-inducing IFNβ). Downstream events involve a number of kinases and adapter proteins that ultimately lead to the activation of the IKK complex [7,9]. Thus, all TLRs elicit conserved inflammatory pathways, culminating, among others, in the activation of NF-κB [7]. $Rela^{-/-}tnfr1^{-/-}$ mice are more susceptible to bacterial infection than $tnfr1^{-/-}$ animals [35]. IKKβ/TNFR1 double knockouts, however, succumb to infection even more rapidly due to a more complete block of the canonical NF-κB pathway [36,37]. In contrast, innate immunity seems to be not affected in $IKK\alpha^{AA}$ mice, where two serines are replaced by alanines in the activation loop pf IKKα making this subunit inactive [38]. Likewise, MEFs from $Ikkg^{-/-}$ mice do not activate NF-κB upon LPS or IL-1 stimulation [39]. Therefore, the innate response activates NF-κB-dependent genes predominantly via the canonical pathway.

3.2. *NF-κB and lymphopoiesis*

It is well established that NF-κB signaling is of outstanding importance during the development and function of the adaptive immune system. The role of NF-κB in this context is described in detail in recently published excellent reviews [9,34]. Therefore, key functions of NF-κB during lymphopoiesis will only be briefly summarized.

Intact NF-κB signaling is crucial during the differentiation steps of T and B cells as well as for their homeostasis. The selection of lymphocytes in the bone marrow and thymus is characterized by a high rate of apoptosis. Gene dissection studies have addressed the question, whether NF-κB is needed for early lymphocyte development: that is before the expression of the pre-T-cell receptor (pre-TCR) or the pre-B-cell receptor (pre-BCR). Hence, the anti-apoptotic functions of classical NF-κB activation play a key role in lymphopoiesis as demonstrated by the observation that combined ablation of p50 and RelA halts lymphocyte development before expression of pre-AgRs [40]. On the biochemical level it was shown that ablation of either IKKβ or IKKγ leads to complete inactivation of classical NF-κB activation [5]. Interestingly, adoptive transfer of classical NF-κB-deficient $ikkb^{-/-}$ fetal liver stem cells has shown that early lymphopoiesis is impaired due

to increased apoptosis of developing lymphocytes [37]. However, mature lymphocytes can be detected upon additional removal of type 1 TNFα receptor, indicating that classical NF-κB signaling prevents premature lymphocytes from TNFα-induced apoptosis [37]. This is in line with data showing that NF-κB acts as prosurvival factor in response to TNFα in early CD34-positive bone marrow cells [41]. The importance of NF-κB for lymphopoiesis and immune responses is strikingly demonstrated in human genetic diseases wherein the gene encoding IKKγ (NEMO) is inactivated by mutation [42]. The *ikkg* gene is located on the X-chromosome and thus is usually subject to random inactivation in individual cells in females. In female patients, however, who are heterozygous for this NEMO mutation, all peripheral lymphocytes exhibit an intact *NEMO* gene. Thus, the absence of peripheral cells with deficient IKKγ-dependent NF-κB signaling indicates an essential, most likely anti-apoptotic function of classical NF-κB activation during early lymphopoiesis [42].

NF-κB fulfills diverse tasks in lymphocyte development that are distinguishable according to the stage before, during or after pre-AgR signaling. There is evidence that expression of pre-AgR leads to survival signals that at least partially depend on NF-κB. For example, T-cell development in the thymus starts with $CD4^-CD8^-$ double-negative (DN) thymocytes, which progress to $CD4^+CD8^+$ double-positive (DP) thymocytes and finally to $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes, which exit the thymus and enter the circulation. All thymocytes have some constitutive NF-κB activity, but the activity is particularly high in later DN thymocytes and essentially promotes the progression to late DN stages via pre-TCR signaling [43]. Notably, ex vivo NF-κB inhibition in isolated late DN cells triggers apoptosis [43] suggesting that NF-κB provides a surviving signal during that stage. It also has to be noted that forced activation of NF-κB by expression of a constitutively active *ikkg* transgene in RAG1 (recombination-activating gene 1)-deficient thymocytes, which cannot assemble the pre-TCR, allows partial progression of thymocytes to the DP stage [43]. Similarly, pre-BCR signaling involves the survival function of NF-κB. The reduced pre-B-cell population that occurs upon expression of the IκBα super-repressor in bone marrow cells can be rescued by overexpression of Bcl-X_L, an anti-apoptotic NF-κB target gene [9]. Moreover, NF-κB signaling is likely to contribute to the initiation of Igκ and Igλ gene rearrangement and thus promotes further B-cell development to immature BCR+ (B-cell receptor-positive) B cells [34]. In immature B cells, however, constitutively activated NF-κB acts as prosurvival factor. Following BCR ligation NF-κB becomes downregulated [34] and cells are sensitized to pro-apoptotic signals during negative selection.

During later stages of thymocyte development it was shown that TCR signals can activate NF-κB and that NF-κB functions in a pro-apoptotic way during negative selection whereas it has anti-apoptotic properties during positive selection [34]. Subsequent to negative and positive selection double-positive thymocytes perform a lineage commitment, become either $CD4^+$ or $CD8^+$ single-positive T cells and exit the thymus.

Conditional gene-knockout experiments are suitable to study the role of NF-κB in later B- and T-cell developmental stages because complete deletion of the proteins of interest occurs only slowly and meanwhile wild-type proteins are functionally expressed. Mice with a conditional deletion of IKKγ lack mutant peripheral T cells, apparently as a consequence of increased apoptosis [44]. Conditional deletion of IKKβ, however, only results in slow reduction of peripheral T cells most likely due to partial compensation by IKKα [44]. Interestingly, peripheral T-cell subsets such as $CD4^+CD25^+$ regulatory T cells (T_{reg}) [44,45], natural killer T (NKT) [45], and memory T cells [44] are clearly reduced upon conditional IKKβ deletion. Taken together, canonical NF-κB signaling is required for long-term survival of T cells from the stage of single-positive thymocytes onwards.

Similarly, late B-cell development requires NF-κB. Upon leaving the bone marrow immature B cells become transitional B cells and finally develop into either follicular or marginal zone B cells. Immature B cells display constitutive NF-κB activity which is negatively regulated upon BCR ligation [34]. In mature B cells, however, it is thought that signaling via BCR fully activates NF-κB which promotes survival [34]. It was shown that ablation of either IKKα, IKKβ or IKKγ/NEMO decreases the numbers of mature B cells in vivo [10,46]. Similarly, p50/p52 as well as RelA/c-Rel double-knockout progenitor cells fail to develop mature follicular and marginal-zone B cells in adoptive transfer experiments with a slightly earlier block in p50/p52 radiation chimeras [24]. Interestingly, Bcl-2 transgene expression not only prevented apoptosis of RelA/c-Rel double-knockout B cells and increased peripheral B-cell numbers, but also induced their further maturation [47]. In this context it has to be noted that BAFF ligation is essential for the survival of transitional and mature B cells by selectively activating the alternative NF-κB pathway with subsequent expression of anti-apoptotic Bcl-2 family proteins in these cells [34]. Hence, both pathways are important for B-cell maturation and survival.

In summary, both classical and alternative NF-κB pathways have crucial functions for the development of immature and maintenance of mature peripheral B and T cells. The primary role of NF-κB in developing and mature lymphocytes probably is to promote survival. Only lymphocytes with intact NF-κB signaling will become part of the peripheral immune system and will contribute to immune answers. Moreover, both pathways are also required for the development of secondary lymphoid organs such as spleen, lymph nodes, Peyer's patches and mucosal-associated lymphoid tissue (MALT) (see Hayden et al. [9] for detailed review). Thus, the role of NF-κB activation in the context of adaptive immune answers cannot be overestimated. Activating the adaptive immune system remains a crucial step for sufficient immune responses and signaling through antigen-specific T-cell (TCR) and B-cell (BCR) receptors is the central event of an adaptive immune response. Activation of NF-κB (primarily canonical) downstream of TCR and BCR ligation is required for the regulation of genes involved in pivotal mechanisms such as proliferation of activated lymphocytes, IL2 production, T_H1/T_H2 differentiation, class switch recombination, and survival (see [9] for details). With intent to therapeutically inhibit proinflammatory NF-κB signaling it has to be kept in mind that the opposite effect can be achieved. That is immunosuppression may be induced which facilitates subsequent invasion of pathogens or

commensal bacterias and thus converts a definitive anti-pathogenic response into an ineffective chronic inflammatory reaction. Given the crucial roles of NF-κB in maintaining immunocompetence, inhibition of NF-κB in order to treat excess activation needs to be carefully investigated in animal models and human diseases to prevent immunosuppression. The goal is to adequately inhibit NF-κB signaling to control inflammation while immunosuppression is prevented.

3.3. *NF-κB and inflammation*

NF-κB is regarded as primary regulator of inflammatory responses. It plays a crucial role in the initiation and amplification of inflammation [4,9,48]. By responding to proinflammatory stimuli such as IL-1β or TNF-α NF-κB activation triggers the transcription of mainly proinflammatory and anti-apoptotic target genes and becomes, at least during an initial phase, part of a positive feedback loop. Prolonged or imbalanced activation, however, is likely to generate chronic inflammation and might favor tumorigenesis [4]. Though the molecular basis of NF-κB activation and its role in the context of immune regulation is now understood in detail, the importance of NF-κB signaling during inflammatory diseases rather derives from indirect evidence. Due to embryonic lethality or defects of immune cell development most, if not all, in vivo models that genetically target molecules of the NF-κB pathways are difficult to study its inflammatory role in diseases. The use of established disease models in these genetically modified animals has to be carefully evaluated and controlled.

However, in vitro, the pivotal proinflammatory function of NF-κB is quite clear. Ablation of the canonical NF-κB pathway leads to a dramatic decrease of inflammatory mediator production upon stimulation in different cell types such as MEFs, macrophages, endothelial cells and lymphocytes [24,49]. Moreover, NF-κB was shown to be involved in the transcriptional regulation of more than 150 genes with a significant portion demonstrating proinflammatory properties [12]. Thus, it can be suggested that NF-κB deficiency or its inhibition in vivo leads to reduced inflammatory responses. Results of in vitro experiments, however, are sometimes contradictory to data derived from in vivo studies. For example, mice adoptively transferred with *rela*$^{-/-}$, *rel*$^{-/-}$c-*rel*$^{-/-}$ or *ikkβ*$^{-/-}$ stem cells as wells as *nfkb2*$^{\Delta C/\Delta C}$ (disruption of the NF-κB2 C-terminal coding region) and *ikba*$^{-/-}$ knockout mice [24,37] demonstrate granulocytosis in vivo. Though NF-κB signaling may directly regulate granulocyte homeostasis in vivo, the observed granulocytosis may be a result of the diminished immune function in these mice. Thus, the granulocytosis can be interpreted, at least in part, as compensatory mechanism to fight opportunistic pathogens. This is in line with data demonstrating that p50-deficient and, more severe, p50$^{-/-}$p65$^{+/-}$ mice develop typhlocolitis under normal housing conditions due to naturally infection with opportunistic pathogens [50]. Recently, two studies were published demonstrating that deficient NF-κB activation in intestinal epithelium is associated with increased inflammation in vivo. Zaph et al. [51] demonstrated that trichuris-stimulated intestinal epithelial cells (IECs) fail to produce the NF-κB-dependent cytokine thymic stromal lymphopoietin (TSLP) in vivo. TSLP has been linked to the induction of T_H2 responses via conditioning of dendritic cell maturation and thus is important for the eradication of parasites such as trichuris [52]. The absence of TSLP, however, favors an inflammatory T_H1 response and undermines a definitive antiparasitic response which finally results in chronic colitits [51]. A similar study by Nenci et al. [53] demonstrated that inhibition of the canonical NF-κB pathway by ablation of NEMO in IECs in vivo reduces the production of defensin-3, an inducible antimicrobial small bowel peptide. As a consequence, enhanced bacterial penetration triggers an inflammatory response and increased TNFα secretion. TNFα causes apoptosis of canonical NF-κB-deficient IECs and thus epithelial barrier function breaks down. Further bacterial infiltration ultimately leads to chronic inflammation [53]. Both studies demonstrate that defects of NF-κB signaling cause immunosuppression which triggers and maintains chronic inflammation. A similar phenomenon is also likely to be evident in humans. Hypomorphic mutations in the X-linked gene encoding NEMO cause a genetic disorder called 'anhidrotic ectodermal dysplasia with immunodeficiency' (EDA-ID) [42]. This disease is caused by short C-terminal truncations or missense mutations of the NEMO protein that leads to reduced activation of NF-κB. The patients are suffering from recurrent infections owing to an impaired innate immune response and may die early in life [54]. More recently, a heterogeneous series of immunodeficient patients were also shown to carry NEMO mutations but did not exhibit any associated signs of EDA [42]. This underscores the central position of canonical NF-κB signaling to mount an appropriate immune answer.

The aforementioned studies describe situations with insufficient NF-κB activation that causes immunosuppression and may result in chronic inflammatory diseases. NF-κB inhibition probably is not helpful in these situations. There are inflammatory diseases, however, that are likely to be primarily based on increased NF-κB activation. For example, a plethora of experimental data assign the NF-κB pathway a causative role during arthritis, asthma, atherosclerosis, diabetes, and other diseases in animal models and humans (Table 1). The

Table 1 – Human diseases with evident NF-κB activation

	Reference
Diabetes	[89]
Atherosclerosis	[59]
Cancer	[4]
Inflammatory bowel disease	[42]
Rheumatoid arthritis	[55]
Gastritis	[90]
Systemic lupus erythematosus	[91]
Asthma	[58]
ARDS	[92]
Sepsis/SIRS	[70]
Surgical major trauma	[93]
Alzheimer's disease	[94]
Amyotrophic lateral sclerosis	[95]
Parkinson's disease	[96]
Multiple sclerosis	[97]

ARDS: acute respiratory distress syndrome; SIRS: systemic inflammatory response syndrome.

canonical NF-κB pathway is constitutively activated in synovial tissue of rheumatoid arthritis (RA) patients [55]. In animal models NF-κB activation leads to the induction of proinflammatory genes and synovial hyperplasia through its anti-apoptotic properties [56]. Interestingly, intraarticular gene transfer of activated IKKβ induces synovial inflammation and RA in rats, whereas intraarticular injection of dominant-negative IKKβ significantly ameliorated the severity of adjuvant arthritis, accompanied by a significant decrease in NF-κB DNA expression [57]. Moreover, as described above in more detail, classical NF-κB signaling is required to protect osteoclasts and their progenitors from tumor necrosis factor α-induced apoptosis and its loss in hematopoietic cells prevents inflammation-induced bone loss [21]. Inflammatory airway disease in humans has also been attributed to increased NF-κB activation as demonstrated by bronchial biopsies from asthma patients [58]. In addition, constitutive NF-κB activity was found in atherosclerotic lesions [59]. NF-κB was further assigned an anti-atherogenic role since macrophage-specific IKKβ deletion increased atherosclerosis in low-density lipoprotein receptor (LDLR)-deficient mice [60]. Moreover, it has been hypothesized that obesity, type 2 diabetes and inflammation are connected. Namely, obese patients exhibit an elevated level of inflammatory cytokines that cause hepatic insulin resistance [61]. By selectively expressing constitutively active IKKβ in hepatocytes Cai et al. [62] were able to induce a type 2 diabetes phenotype in mice, characterized by hyperglycemia, profound hepatic insulin resistance, and moderate systemic insulin resistance. Deletion of hepatic Ikkβ, however, retains liver insulin responsiveness but insulin resistance in muscle and fat in response to high fat diet is developing [63]. In contrast, mice with IKKβ ablation in myeloid cells retain global insulin sensitivity and are protected from insulin resistance [63]. These findings indicate that lipid accumulation in the liver leads to subacute hepatic 'inflammation' through NF-κB activation and downstream cytokine production with subsequent insulin resistance both locally in liver and systemically. Thus, inhibition of canonical NF-κB activation or IKKβ activity, especially in myeloid cells, may in theory be used to treat insulin resistance.

The facts discussed so far imply that proinflammatory NF-κB signaling should not be inhibited under basal conditions or during mild forms of activation to prevent immunosuppression. From this therapeutic point of view, however, it has to be noted that exclusively increased constitutive activity of NF-κB in certain organs is sufficient to cause inflammation. For example expression of constitutive active IKKβ via intratracheal administration of an adenoviral vector leads to increased NF-κB activation, inflammatory mediator production, and neutrophilic lung inflammation [64]. This non-infectious, 'spontaneous' lung inflammation was prevented by coadministration of adenoviral vectors expressing a transdominant inhibitor of NF-κB demonstrating that the observed inflammatory effects were only dependent on NF-κB activation [64]. Similarly, doxycycline regulated, conditional expression of constitutively active or dominant-negative IKKβ in acinar cells in vivo was sufficient to cause acute pancreatitis or ameliorated cerulean-induced pancreatitis, respectively [65].

These studies suggest a beneficial effect of NF-κB inhibition for example during hyperinflammatory diseases. LPS-induced systemic inflammatory response syndrome (SIRS) is an appropriate model to study a systemic and hyperinflammatory role of NF-κB signaling. Moreover, this model facilitates the investigation of systemically administered NF-κB inhibitors. NF-κB is readily activated upon intraperitoneal LPS challenge within 4 h in lung, liver and spleen followed by significant increases of proinflammatory cytokines and chemokines [66]. Similar results were obtained by means of in vivo bioluminescence imaging of NF-κB-dependent luciferase expression in transgenic mice upon intraperitoneal LPS and luciferin treatment [67]. In these models, NF-κB inhibition can be beneficial. For example, LPS-induced myocardial dysfunction is associated with NF-κB activation in cardiomyocytes and can be prevented in transgenic mice expressing a non-degradable form of the NF-κB inhibiting protein IκBα [68]. Notably, most, if not all, pathophysiological mechanisms that can induce acute cellular stress responses and subsequent inflammation are likely to activate NF-κB with a focus on the canonical pathway (reviewed in [69]). Experimental data on the role of alternative NF-κB activation are rather scarce. However, it was shown recently that in macrophages classical NF-κB signaling is negatively regulated via the alternative pathway [20]. Compared to wild-type mice $Ikka^{AA/AA}$ mice reveal increased inflammatory responses and bacterial clearance but decreased survival rates upon systemical group B streptococcus challenge. This is due to IKKα-dependent suppression of NF-κB activity by accelerating both the turnover of the NF-κB subunits RelA and c-Rel, and their removal from proinflammatory gene promoters which ultimately contributes to the resolution of inflammation [20].

Evidence of the role of NF-κB during systemic hyperinflammation in humans is rather limited. Böhrer et al. describe a highly significant difference of increased NF-κB activity in peripheral blood mononuclear cells of septic patients with fatal outcome compared to survivors [70]. Similar results of increased NF-κB activation in mononuclear cells of non-survivors were obtained in patients who fulfilled the criteria for non-infectious systemic inflammation [71]. This prognostic value of NF-κB activity in mononuclear cells was confirmed by a larger study in septic patients [72]. Increased NF-κB activation was also found in broncho-alveolar lavage cells of patients after bone marrow transplantation and during adult respiratory distress syndrome (ARDS), in mononuclear cells of patients with acute diseases such as appendicitis, and in neutrophils of aortic aneurysm surgery patients (reviewed in [69]). There is a positive correlation between an increased overall transcriptional activity of NF-κB and the severity and development of fatal complications of the particular disease in all these studies. These data are in line with a more recent study that examined the role of two haplotypes of the *irak-1* (IL-1 receptor-associated kinase) gene in septic patients [73]. IRAK-1 plays a central role in TLR2- and TLR4-induced activation of NF-κB during the innate immune response. The IRAK-1 variant haplotype was associated with increased NF-κB activation in LPS-stimulated peripheral blood neutrophils from septic patients compared to patients with wild-type IRAK-1 haplotype. Moreover, patients with the variant IRAK-1 haplotype demonstrated an increased incidence of septic

shock and significantly reduced survival rates [73]. Hence, the magnitude of NF-κB activation directly correlates with an adverse course of systemic inflammatory diseases such as sepsis. Consequently, NF-κB inhibition may be beneficial, at least during certain disease phases.

There are several other human autoimmune or fever diseases that are associated with increased NF-κB activation, such as Mediterranean fever, Crohn's disease or asthma (for detailed review see [42]). Genetics are likely to play an important role. Thus, these diseases can be caused by mutations in genes that modulate NF-κB activity, polymorphisms of NF-κB signaling molecule genes or promoter/enhancer κB site mutations in NF-κB target genes. Mutations of genes that are involved in negative regulation of NF-κB seem to play an important role in this context [42].

In conclusion, the anticipated role of NF-κB as a central regulator during acute stress and inflammatory responses seems to apply particularly during systemic inflammation in humans. A basic pathophysiological concept consitutes that classical NF-κB signaling promotes hyperinflammation via positive feedback mechanisms and uncontrolled production of proinflammatory cytokines. However, more recent experimental data [74] showed an anti-inflammatory function of canonical NF-κB in addition to the anti-inflammatory function of IKKα in macrophages as described above. Thus, the authors demonstrated that NF-κB activation in leukocytes recruited during the onset of inflammation is associated with pro-inflammatory gene expression in a rat pleuritis model, whereas late activation during the resolution of inflammation is associated with the expression of anti-inflammatory genes and the induction of apoptosis. Inhibition of regular NF-κB activation during the resolution of inflammation protracts the inflammatory response and prevents apoptosis of inflammatory cells. This suggests that NF-κB has an anti-inflammatory role in vivo involving the regulation of inflammatory resolution [74].

Accordingly, it remains to be elucidated which function of NF-κB – its proinflammatory (via classical activation) or its anti-inflammatory (partially modulated via the alternative pathway) properties – is pathophysiologically and therapeutically more relevant during inflammation. This issue, however, could be addressed by using selective inhibitors of IKK subunits.

4. NF-κB inhibition to control inflammation: a double edged sword

4.1. *Strategies to therapeutically modulate NF-κB activation*

Recently, NF-κB signaling and pathways that regulate its activity have become a focus for intense drug development and application screening. As the molecular mechanisms of the NF-κB pathway are analysed now in detail several different strategies for inhibiting NF-κB activation or function are conceivable and have been evaluated (Fig. 1) (for details see [75,76]). The different ways of inhibiting NF-κB signaling can be distinguished according to the degree of specificity. Relatively unspecific inhibition of NF-κB activation happens through inhibitors that act upstream the IKK complex. That is, for example, blockade of the TNF receptor or inhibition of TRAF adaptor molecule function. Similarly, blockers of the ubiquitin-proteasome pathway suppress activation of NF-κB by stabilizing IκB. Inhibitors of the 26S proteasome for example are known to block IκBα degradation and NF-κB nuclear translocation as well as inducible p100 processing and have entered clinical development [75]. Nonetheless, these approaches are unlikely to result in a very specific NF-κB blockade.

A higher degree of specificity may be achieved by direct inhibition of NF-κB-specific transactivation. A heterogenic group of inhibitors consists of compounds that downregulate one or more steps of NF-κB nuclear functions which involve nuclear translocation, DNA binding and transcriptional activation. For example, a cell-permeable peptide that contains the nuclear localizing sequence of p50 is thought to inhibit nuclear translocation of p50-containing dimers by saturating the nuclear import machinery. Several peptides of this type have been described, the most commonly used one is the SN50 peptide [76]. However, SN50 not only acts on NF-κB signaling, it also prevents nuclear translocation of AP-1, NFAT and STAT1 [77]. Hence, the anti-inflammatory and antitumor activity induced by distinct peptides such as SN50 and observed in vivo is not NF-κB specific but blocks nuclear translocation of a number of non-NF-κB transcription factors as well.

Another effective and often used approach of inhibiting NF-κB nuclear transport and DNA binding is accomplished by using mutant forms of IκBs (super-repressors, SRs), which cannot be phosphorylated or degraded. Thus, these mutant IκBs stably bind to NF-κB and prevent its activation. Commonly, in IκBα-SRs Ser 32 and 36 are exchanged with alanines [19] but other stable mutant forms of IκBs are known as well [76]. These IκB-SRs have been used successfully in several in vivo transgenic models to study the developmental role of NF-κB signaling in certain tissues (see Gerondakis et al. [78]). Of note, because IκBα does not bind to RelB-containing complexes the IκBα-SR is supposed to be primarily a blocker of the canonical pathway.

A large class of non-specified NF-κB inhibitors block DNA binding and κB site-dependent gene expression. This group contains sesquiterpene lactones (SLs) such as parthenolide which directly prevent NF-κB DNA binding through interaction with cysteine residues in the DNA binding loop of rel proteins [79]. Various SLs have been demonstrated to block inflammatory responses in vivo which furthermore inhibits inflammation-induced tumorigenesis via prevention of metastasis and induction of apoptosis [80]. Another molecular method to block specific NF-κB DNA binding can be achieved by means of κB site decoy oligodeoxynucleotides (ODNs). ODNs interfere with the binding of NF-κB to promoter regions resulting in downregulation of target genes. A number of animal studies has shown anti-inflammatory properties of such decoy oligonucleotides [81]. However, ODNs are degraded rapidly in vivo and therefore high doses of ODNs have to be delivered frequently which prevents their clinical use so far. In addition, NF-κB signaling may be suppressed by means of RNA silencing, but this approach has to be evaluated more precisely in vivo. There is limited experimental evidence in vitro that

the final step of NF-κB signaling, namely transcriptional activation, may be blocked by certain molecules such as D609, RO31-8220 and SB203580 which normally are known as selective blockers of protein kinase C and p38 MAPK. These and other substances are thought to prevent phosphorylation of NF-κB subunits like RelA leading to reduced transactivation [76]. The anti-inflammatory effects of these compounds with respect of NF-κB signaling need to be evaluated in vivo.

As IKK integrates many NF-κB-activating pathways, the most effective and specific approach to modulate NF-κB activity might result from IKK inhibitors. However, unspecific IKK inhibitors can be separated from more specific substances. NSAIDs such as aspirin, sulindac, and sulphasalazine are known to exert their NF-κB blocking effects independently of COX inhibition but, at least in part, through inhibition of IKK-mediated phosphorylation of IκBα [82,83]. However, NF-κB pathway inhibition by theses substances is likely to occur at multiple steps which restricts specificity. Similarly, other immunmodulatory substances such as thalidomide and analogues, cyclopentenone prostaglandins (cyPGs) and antioxidants (e.g. vitamin C, flavenoids) have been shown to prevent IκBα phoshorylation by IKKβ [75]. It is further important to note that antioxidants such as *N*-acetyl-L-cysteine and pyrrolidine dithiocarbamate (PDTC) can also inhibit the activity of other components of NF-κB signaling pathways, including TNF receptors and the proteasome, independently of direct effects on IKK, which shortens their specificity [75,76]. An interesting specific approach of canonical NF-κB inhibition was shown by using the IKKγ/NEMO subunit of the IKK complex as a target. A peptide corresponding to the NEMO-binding domain (NBD) of IKKβ and fused with a cell permeability domain can be used as IKK inhibitor [84]. Thus, NEMO binding to IKKβ and regular complex formation is prevented resulting in inactivation of canonical NF-κB signaling. The NBD peptide was shown to inhibit inflammation in vivo in several murine disease models such as inflammatory bowel disease, experimental allergic encephalomyelitis (a model of multiple sclerosis), inflammatory bone disease, and arthritis (for details see [76]). These investigations once more underline the crucial role NF-κB plays in the signaling cascade leading to organ inflammation.

Most promising are selective IKK inhibitors which are reviewed more precisely elsewhere [75]. Up to now, 14 compounds are described with predominant IKKβ inhibiting specificity due to the fact that the canonical pathway has been the center of scientific focus for a long time. With now increasing understanding of the physiological role of the alternative pathway, however, the development of specific IKKα inhibitors can be expected. Those compounds are supposed to be helpful in the treatment of diseases involving the adaptive immune system such as autoimmune diseases or cancer [38,75]. Most of the IKKβ inhibitors that have been made public so far, however, are characterized regarding their pharmacokinetics and dynamics in cell-based assays and were demonstrated to exert their function via ATP competition. Interestingly, several of them have shown to inhibit TNFα production upon LPS stimulation in murine models [75]. Most recently, two selective IKKβ inhibitors have been studied more extensively in vivo. Oral administration of ML120B (Millenium Pharmaceuticals) in mice was shown to inhibit NF-κB activation in different organs such as lung, liver and lymphoid organs upon LPS stimulation. NF-κB inhibition in bone marrow and spleen is associated with clear apoptosis and leads to rapid TNF-dependent depletion of B and T cells [85] and thus confirms earlier data in genetically modified mice [37,40]. Another selective IKKβ inhibitor, compound A (Bayer Yakuhin, Ltd.), was tested in several non-infectious inflammatory models in rodents [86]. The authors did not report on similar lymphopoietic defects but clearly demonstrated anti-inflammatory effects in vivo [86]. Taken together, these studies demonstrate both potential benefits and drawbacks of selective IKKβ inhibitors. It might be assumed that pharmacological suppression of inflammation-induced IKKβ and subsequent NF-κB activity would leave some NF-κB-dependent signaling and is beneficial during inflammatory sates. Ongoing or excessive inhibition, however, may later cause lymphopenia and immunosuppression due to blockade of basal NF-κB activity. Thus, the results of these studies are promising for further development and investigations of IKKβ inhibitors in clinical trials.

4.2. *Anti-inflammatory NF-κB inhibition: when?*

There is no doubt that the NF-κB signaling cascade is a key initiator of inflammation and thus constitutes a promising target of anti-inflammatory therapeutic strategies. However, appropriate indications of NF-κB inhibition with respect to distinct disease courses and clinical situations need to be determined. In particular because NF-κB activation is a very dynamically regulated process, the time course of NF-κB activation has to be evaluated precisely in certain diseases. Given the physiological roles of NF-κB signaling that occur in parallel and subsequent to the activation of proinflammatory genes such as resolution of inflammation and anti-apoptosis, inadequate NF-κB inhibition might result in chronic inflammation or cell death.

The dilemma of NF-κB inhibition is clearly demonstrated by Chen et al. [87] who showed a dual function of the IKK/NF-κB signaling pathway using a murine ischemia–reperfusion model. By ablation of the β-subunit of IKK in enterocytes the authors were able to specifically block classical NF-κB activation in the intestinal epithelium. Reperfusion usually culminates in an systemic inflammatory response and in multiple organ dysfunction in this model. This was suppressed upon intestinal IKKβ deletion. Interestingly, in contrast to the diminished systemic inflammation, local IKKβ deficiency results in severe apoptotic damage to the reperfused intestinal mucosa. Obviously, one crucial function of IKK and NF-κB signaling is to provide protection from stressfull challenges. However, the protection enabled by NF-κB-induced gene expression goes along with potential harmful local and systemic inflammation. Inflammation and apoptosis are known to be opposing processes, and by inhibiting apoptosis, IKKβ promotes inflammation. Although NF-κB pathway inhibition is likely to be a potent anti-inflammatory approach in vivo, this study underscores its potential adverse effects. Thus, tissue or cell-specific inhibitors that target the NF-κB pathway in inflammatory cells but not in epithelial cells would constitute a better intervention mode. So far, however, this remains a desire which is not likely to reach the clinic in the near future.

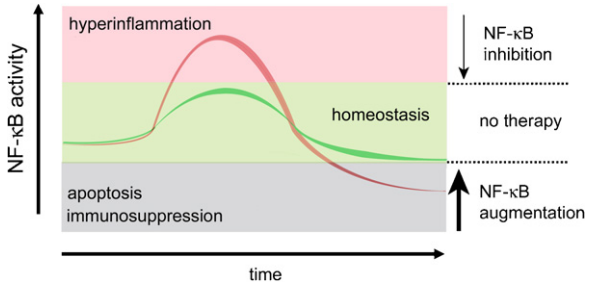

Fig. 2 – Proposed hypothetic scheme of NF-κB activity kinetics in tissues and impact on inflammation and apoptosis. Appropriate initial NF-κB activity regulates 'normal' inflammation and is followed by negative NF-κB regulation which favors apoptosis and resolution of inflammation, respectively. Excess activation of NF-κB, for example during massive infection in septic shock, results in hyperinflammation where inhibition of NF-κB signaling is beneficial. It is conceivable that massive NF-κB activation may induce intense NF-κB downregulation, at least in certain tissues such as immune cells. This ultimately causes immunosuppression and fatal outcome. Here, stimulation of the NF-κB pathway may be helpful.

Taken together, as long as tissue-specific therapies are not possible, systemic hyperinflammation constitutes a pathophysiological situation where inhibition of the NF-κB pathway is likely to be a promising therapeutic strategy at present. It occurs during acute severe diseases such as shock, major trauma or sepsis where the inflammatory response is generalized. Moreover, its magnitude determines the outcome. As already discussed above, NF-κB activation is likely to play a key role in this scenario [70,72]. Thus, from the clinical point of view systemic hyperinflammation is an appropriate situation where NF-κB inhibition should be evaluated in clinical trials. However, NF-κB inhibition should be performed with caution especially during situations when NF-κB is not excessively activated. Notably, inflammatory responses in vivo are very dynamically regulated and this may be reflected by the activity of NF-κB on a molecular level (Fig. 2). For example, a typical septic course in patients is characterized by initial hyperinflammation followed by a compensatory anti-inflammatory response with compromised immune answers and low lymphocyte counts due to apoptosis [88]. Therefore, initial NF-κB inhibition may be helpful whereas late blocking may aggravate immunosuppression and worsen the outcome. Indeed, in a murine sepsis model augmentation of NF-κB activation in lymphocytes is associated with increased lymphocyte numbers and improved survival rates (U. Senftleben et al., manuscript in preparation).

In summary, it turns out that a balanced NF-κB activity/activation is needed to maintain immune homeostasis and to regulate appropriate inflammatory responses. Thus, it has to be emphasized that adequate inhibition of NF-κB signaling requires routine monitoring of NF-κB activity to prevent adverse effects of complete inactivation. Likewise, if NF-κB should be inhibited in patients with arthritis or inflammatory bowel disease where systemic NF-κB activity is supposed to be 'normal', this therapy should be guided by monitoring the patient's immune status. The development of such routinely applicable methods as well as the evaluation of specific NF-κB inhibitors during certain diseases is an exciting field for future research.

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