CHAPTER 2

CONSTITUTIVE NF-κB ACTIVATION IN AML: CAUSES AND TREATMENT STRATEGIES

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Resistance to apoptosis is regarded as one of the hallmarks in the process of leukemogenesis. The transcription factor NF-κB is activated by various cytokines that stimulate cell survival and growth; consequently, NF-κB drives the expression of multiple proliferative and anti-apoptotic genes. For more than a decade, it has been known that NF-κB is constitutively activated in a majority of acute myeloid leukemia (AML) patients. Inhibition of NF-κB has been shown to induce apoptosis in AML cells, but the clinical effectiveness of NF-κB inhibitors has been inadequate. In recent years, possible causes underlying this continuous NF-κB activity have been elucidated. It has been shown that chromosomal translocations or mutations leading to development of leukemia drive the increase in NF-κB activity. Furthermore, autocrine/paracrine cytokine signaling and increased expression of NF-κB signaling components play an important role in the continuous NF-κB activation. Moreover, high proteasome activity is often observed in AML patients. Whereas NF-κB activity is dependent on the proteasome, enhanced activation of the proteasome also results in constitutive NF-κB activity. In the present study, we described these underlying molecular mechanisms leading to constitutive NF-κB activity and discussed the novel treatment strategies based on the inhibition of NF-κB activation.

2.1 NF-κB SIGNALING

2.1.1 ACTIVATION OF THE NF-κB COMPLEX

The transcription factor nuclear factor κB (NF-κB) is a protein complex which controls the gene expression of genes involved in inflammation and the immune response. Whereas NF-κB induces the expression of various genes involved in proliferation and anti-apoptosis, NF-κB activity has also been recognized as playing a major role in leukemia [1]. NF-κB can be activated by diverse stimuli including cytokines, such as TNFα, IL-1β and Toll-like receptor 4 (TLR-4) [2]. Consequently, a variety of genes are regulated by NF-κB, such as the anti-apoptotic proteins (cFLIP, BCL-2 and BCL-XL), growth factors, cytokines (IL-1 and IL-6), cell adhesion molecules and chemokines [3]. Importantly, only a subset of genes are activated by NF-κB in a stimulus and cell-specific manner. NF-κB complexes are homo- or heterodimers formed by members of the Rel family, which consists of five proteins: p65 (RelA), RelB, c-Rel, p50 and p52. Two major signaling pathways can activate NF-κB, the canonical NF-κB pathway and the non-canonical pathway (Figure 2.1). The canonical pathway can be induced by a variety of cytokines including IL-1, TNFα, and TLR-2/4. Upon binding of IL-1 to the IL-1 receptor (IL-1R), the IL-1R accessory protein (IL-1RAP) and MYD88 form a complex with the IL-1 receptor. This results in the recruitment of the IL-1 receptor associated kinase (IRAK) and TNF-receptor associated factor (TRAF) proteins to the complex. Similarly, binding of TLR-2/4 to the Toll-like receptor (TLR) also induces the association with IRAK and TRAF. Association of TNFα to the TNF receptor leads to the recruitment of receptor-interacting serine/threonine protein kinase (RIPK) and TRAF to the receptor.
Figure 2.1: Canonical and non-canonical NF-κB signaling

Activation of these complexes induces a kinase cascade leading to the activation of NF-κB [4, 5]. Firstly, TGF-β activated kinase 1 (TAK1) is phosphorylated at multiple sites including threonine 178, 184 and 187 and serine 192. Phosphorylation of threonine 184 and 187 is mainly necessary for optimal NF-κB activation [6]. Binding of TAK1 to the TAK1-binding protein 1 (TAB1) and TAK1-binding protein 2 or 3 (TAB2/TAB3) is also required for full activation of TAK1 which then activates the IκB kinase (IKK) complex [7–9]. This complex consists of IKKI/IKKα, IKK2/IKKβ and NEMO/IKKγ [10]. TAK1-mediated phosphorylation of IKK2 results in activation of the NF-κB complex by phosphorylation of the inhibitory NF-κB protein IκB. IκB proteins, such as IκBα and IκBβ, bind to the nuclear localization domain of the NF-κB complex and thereby prevent nuclear translocation and NF-κB mediated gene expression [11]. Upon phosphorylation of specific serines of IκB (e.g. in the case of IκBα serine 32 and 36), the IκB protein will be ubiquitinated and subsequently degraded by the proteasome [12, 13]. This directly results in the activation of the canonical NF-κB complex p65/p50.

The non-canonical pathway mainly plays a role in the development of B and T lymphocytes and can be activated by a small number of stimuli, including B-cell activating factor (BAFF), CD40L, receptor activator for NF-κB ligand (RANKL) and lymphotoxinβ. Subsequent NIK activation results in the phosphorylation and activation of the IKKI dimer. Thereupon, p100 is phosphorylated on serines 886
and 870 by IKK1, which leads to the ubiquitination and partial degradation by the proteasome to p52 [14]. This results in the activation of non-canonical NF-κB complex p52/RelB, ultimately leading to the expression of the target genes.

2.1.2 NF-κB-MEDIATED GENE TRANSCRIPTION

Upon activation and translocation to the nucleus of the various NF-κB complexes, these dimers undergo multiple modifications, which subsequently lead to gene transcription (Figure 2.2). Phosphorylation on serine 276 and serine 536 of p65 is mediated by protein kinase A (PKA) and IKK, and is required for the binding of p65 to the CBP/p300 complex [15, 16]. The CBP/p300 complex consists of CBP and p300, which associate with various transcription factors and connect them together on sequence specific loci [17]. Furthermore, the CBP/p300 complex has intrinsic histone acetyltransferase (HAT) activity, leading to acetylation of histones such as H3K27, but it is also able to acetylate proteins like p65 [17, 18]. Upon phosphorylation of p65, CBP/p300 mediates acetylation of p65 on various lysines (e.g. K218, K221 and K310), thereby increasing the DNA binding affinity, decreasing the association with the negative regulator IκBα and promoting the association of the NF-κB complex with bromodomain 4 (BRD4). This results in NF-κB transactivation and gene transcription [15, 19]. BRD4 belongs to the bromodomain and extraterminal domain (BET) family which are involved in transcriptional coactivation and elongation [20]. Acetylated histones and proteins such as p65 are recognized by the BET domain of BRD4 [21] leading to the recruitment of pTEFb [22, 23] and chromatin remodeling complexes like the SWI/SNF complex [24], subsequently leading to activation of gene transcription. Thus, acetylation of p65 by CBP/p300, acetylation of histones by acetyltransferases like CBP/p300 and the recognition of the acetylation marks by BRD4 may be crucial steps in the transcription of genes.
regulated by NF-κB.

Selectivity of expression of NF-κB target genes is at least partially mediated by the affinity and specificity of each dimer towards the so-called κB-DNA binding site (GGGRnY, R=A/G, Y=T/C). Hereby, each stimulus can activate a group of genes in a cell-specific manner. For example, it was recently shown that IL-1 induced the recruitment of p65 to more than 400 enhancers in a TAK1-IKK2 dependent manner, which resulted in the recruitment of PolIII, Pol(S5)II, AP-1, CBP and p50 at those sites [25].

2.2 NF-κB activity in normal hematopoiesis

Basal NF-κB activity is required for HSCs self-renewal and differentiation into myeloid and lymphoid lineages. The quality of HSCs was decreased upon deletion of the canonical and non-canonical NF-κB complexes, p65 and RelB/p52 [26, 27]. Knockout of p65 or double knockout of RelB/p52 resulted in impaired engraftment and self-renewal of HSPCs. In addition, differentiation efficiency of HSCs was affected in p65 knockout mice, whereas deletion of IκBα increased the differentiation of hematopoietic cells, suggesting that NF-κB contributes to the differentiation process. In particular, NF-κB plays a key role in the differentiation and activation of monocytes and granulocytes [28, 29]. Deletion of NF-κB regulators upstream of these NF-κB complexes appeared to be even more severe, as these components also regulate other survival-related pathways. Besides the phosphorylation of IκBα and subsequent NF-κB activation upon TNFα stimulation, IKK is also able to phosphorylate and inactivate the pro-apoptotic protein BAD on serine 32 [30]. In addition, deletion of TAK1 resulted in a rapid induction of cell death of normal HSCs, likely due to simultaneous inhibition of the NF-κB, JNK, p38 and ERK pathways [31]. This effect is largely mediated by TNFα, as the phenotype of TAK1 knockout mice is partially rescued by knockout of both TNF receptors [32, 33]. These findings indicate that a basal NF-κB activity is required for proper functioning of the hematopoietic stem and progenitor cells.

2.3 NF-κB activity in AML

Resistance to cell death and sustained proliferative signaling are hallmarks of cancer development [34]. NF-κB signaling contributes to these biological process, so constitutive NF-κB activity could result in increased proliferation and survival of AML cells. For 15 years it has been known that NF-κB is constitutively activated in the AML stem cell-enriched CD34+ population cells in a large percentage of patients [35–37]. Constitutive activation of NF-κB has also been detected in various murine AML models, including murine GMPs cells transformed by the retroviral transduction of AML1-ETO, BCR-ABL, MLL-ENL and MOZ-TIF2 [38]. In particular, leukemia-initiating cells are marked by high NF-κB activity. The highest IKK activity and NF-κB activation is found in M4 and M5 AML subtypes compared to M1 and M2 subtypes, suggesting that NF-κB is mainly increased at the monocyte
differentiation stage [35]. This finding is in line with the role of NF-κB in normal hematopoiesis, in which monocyte differentiation and activation coincide with high NF-κB activity.

Even though NF-κB activity is increased in many AML patients, increased NF-κB signaling does not alter steady-state hematopoiesis. Overexpression of constitutive active IKK or p65 in cord blood CD34+ cells did not affect the proliferation, differentiation or self-renewal of these cells [39]. These findings suggest that increased NF-κB activity is mainly required for the maintenance of AML cells, but does not play a major role in myeloid transformation.

More importantly however, initial results with the proteasome inhibitor MG132 and IKK2 inhibitor AS602868 showed that targeting NF-κB provoked cell death in AML cells in vitro and in vivo, whereas normal bone marrow CD34+CD38- cells were less sensitive to NF-κB inhibition [37, 40, 41]. This indicates that AML cells are likely more dependent on NF-κB signaling for their survival than normal bone marrow cells. Therefore, NF-κB was proposed as an attractive candidate to be targeted in AML. Since then, many studies have shown the important function of NF-κB in AML cell survival and the potential role of NF-κB as a target in AML.

In the following subsections we discuss the various routes that have been proposed as being involved in the constitutive activation of NF-κB. Furthermore, we highlight how the interference in these pathways could potentially affect AML cell survival.

2.3.1 NF-κB ACTIVATION AS DIRECT CONSEQUENCE OF THE CHROMOSOMAL TRANSLOCATION/MUTATION

Amplifications or rearrangements of RelA, RelB, c-rel, NF-κB1 and NF-κB2 or mutations in IkBα are often found in lymphomas, lymphoid leukemias and Hodgkin’s disease [5, 42]. However, genetic alterations of these NF-κB genes have not frequently been found in myeloid leukemias, suggesting that sustained activation of NF-κB is caused differently in AML cells than in lymphoid malignancies [14, 43]. One of the causes of constitutive activation of NF-κB is directly related to the initial chromosomal translocation or mutation in the myeloid leukemias (Table 2.1).

In normal hematopoietic cells it has been shown that the c-terminal region with intact RUNT-domain of AML1 binds to IKK, thereby inhibiting the kinase activity of IKK [44]. However, the AML1-ETO fusion product, which lacks the c-terminal region of AML1, was no longer able to bind to IKK, which subsequently resulted in NF-κB activation. Inhibition of NF-κB by the proteasome inhibitor bortezomib induced cell death of transformed AML1-ETO cells, showing that maintenance of these AML1-ETO cells is dependent on NF-κB signaling. Moreover, the transcription factor AML-1 induced the expression of the microRNA mir-223 [45, 46], which repressed the expression of IKK1 [47]. In contrast, the fusion product AML1-ETO also binds to the mir-223 promoter, upon which histone proteins are aberrantly deacetylated and CpGs are methylated, resulting in altered chromatin packaging [45]. In this context, AML1 is no longer able to bind to the mir-223 promoter, leading to decreased expression of mir-223 and likely to elevated levels
Table 2.1: Genetic aberrations leading to increased NF-κB activation

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<th>Genetic abnormalities</th>
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| AML1-ETO translocation | 1) Increased IKK activation by inhibition of AML1 association to IKK  
                                      2) Increased IKK expression by repression of microRNA-223 |
| C/EBPα mutation       | 1) Binding to p50 promoter  
                                      2) Increased IKK expression by repression of microRNA-223 |
| 5q deletion           | Increased TRAF6 and p62 expression by loss of mir-146 |
| BCR-ABL translocation | Increased IKK2 activation by activation of PRKD2 |
| FLT3-ITD mutation     | 1) Increased TAK1 activation  
                                      2) Increased IKK2 activation |
| MOZ-TIF2 translocation| Unknown |
| MLL translocations    | Unknown |

of IKK1. The transcription factor C/EBPα also induced the expression of mir-223, whereas C/EBPα mutant proteins failed to express mir-223 [48], possibly also resulting in increased expression of IKK. Nevertheless, it remains unknown whether loss of mir-223 in AMLs carrying the AML1-ETO fusion products or C/EBPα mutants is directly related to decreased expression of IKK. On the other hand, C/EBPα and C/EBPα mutants could further increase NF-κB activity by binding to the p50 promotor, thereby elevating the expression of p50. Moreover, p50 regulates the expression of C/EBPα, thus inducing a positive feedback loop between C/EBPα and p50. It has also been shown that C/EBPα or C/EBPα mutants physically interact with p50 at the promoter site of the anti-apoptotic genes BCL-2 and C-FLIP [49–52]. Targeting this interaction might therefore abrogate the expression of these genes and induce apoptosis in C/EBPα mutant AML cells.

Deletion of chromosome 5q also resulted in increased NF-κB activity. MicroRNA mir-146, which resides on band q34, repressed the expression of TRAF6 and IRAK1 [53–55]. Recently, it was shown that loss of mir-146 in del(5q) high-risk MDS/AML cells resulted in increased expression of TRAF6 in AML cells [56]. Increased NF-κB signaling led to overexpression of p62, a scaffold protein of TRAF6. The positive feedback loop then mediated the constitutive activation of NF-κB in these del(5q) AML cells. However, whether loss of mir-146 also depresses the expression of IRAK1 has not yet been investigated.

Constitutive activation of NF-κB also occurs in a BCR-ABL dependent manner. High NF-κB activity has been observed in Ph+ CML blasts [57]. Moreover, transduction of DA1 cells [58], Ba/F3 cells [57–59] or murine derived bone marrow cells [38, 60] with BCR-ABL resulted in elevated NF-κB activity. It has been proposed
Whereas these cytokines can stimulate their own expression via NF-κB activation, BCR-ABL interacts with protein kinase D2 (PRKD2) and activates PRKD2 by tyrosine phosphorylation [61]. PRKD2 is then able to activate NF-κB by phosphorylation of IKK2 [61], but not IKK1 [57, 61], resulting in the phosphorylation and degradation of IκBα. The role of NF-κB in this type of leukemia is also supported by the finding that genetic targeting of NF-κB or pharmacologic inhibition by anti-TNFα antibody both induced cell death in BCR-ABL transformed cells [60, 62].

In addition, it has been observed that overexpression of FLT3 or activating mutations of FLT3 (e.g. FLT3-ITD), which are both found in AML, trigger the NF-κB pathway [36, 63]. FLT3-ITD enhanced the phosphorylation and activation of TAK1, thereby activating the non-canonical NF-κB pathway [64]. Besides this mechanism, direct interaction between FLT3 and IKK2 has also been observed. Binding of FLT3 to IKK2 resulted in phosphorylation and activation of IKK2, which induced the canonical NF-κB pathway [65]. Contradictory results have been reported regarding the suppression of constitutive NF-κB upon FLT3 inhibition. One study reported that addition of a FLT3 inhibitor induced apoptosis in a subset of high-risk MDS and AML cells, which coincided with a diminished NF-κB activity [65]. In contrast, a different study showed that constitutive activation of NF-κB was not changed upon treatment with a FLT3 inhibitor [36]. However, in that study no information was given regarding the cytotoxic effects in these AML cells.

As mentioned previously, MOZ-TIF2 and MLL-fusion products might also regulate the activity of NF-κB [38]. However, the mechanism underlying the constitutive activation of NF-κB by the fusion products is not known. Nonetheless, NF-κB plays an important role in leukemic transformation mediated by MLL-fusion proteins. It has been proposed that NF-κB activity is required for the recruitment of MLL oncoproteins to the HOXA9/MEIS1 loci in murine transformed cells [66]. Knockdown of p65 induced cytotoxic effects in MLL-rearranged leukemias in vitro and significantly extended the survival of mice. However, whether the epigenetic effect of MLL is also dependent on NF-κB in primary rearranged leukemias has not yet been investigated.

2.3.2 AUTOCRINE/PARACRINE SIGNALING INDUCES CONSTITUTIVE NF-κB ACTIVITY

For several decades, it has been known that a proportional subset of AMLs express high levels of various cytokines, including TNFα, IL-1 and IL-6 [67]. In line with these findings, murine BM cells transduced with the MLL-AF10 oncogene also expressed high levels of TNFα and IL-1 [68]. Secretion of TNFα also induces AML growth in an autocrine-dependent manner [67]. Moreover, it synergizes with IL-3 and GM-CSF to stimulate cell growth by upregulation of IL-3 and GM-CSF receptors [69, 70]. Likewise, IL-1 secretion by AML cells has been observed, which stimulated AML cell growth in an autocrine-feedback loop [67, 71–74]. IL-1 did this not only by stimulating the expression of GM-CSF by AML cells, but also by stimulating the production of GM-CSF and G-CSF by endothelial cells [73, 75]. Whereas these cytokines can stimulate their own expression via NF-κB activation,
it is plausible that these cytokines contribute to the constitutive activation of NF-κB observed in a subset of AML patients. Indeed, IL-1 and IL-6 secretion was associated with NF-κB activity in primary AML cells [76]. Similarly, it has been shown that constitutive NF-κB activation in the murine MLL-ENL, MOZ-TIF2 or BCR-ABL/NUP98-HOXA9 AML models and human CML cells is dependent on autocrine TNFα signaling [38, 62]. Furthermore, it has been shown that TNFα expression and secretion was mediated via activation of NF-κB, thus illustrating the positive feedback loop between TNFα and NF-κB. Nevertheless, constitutive NF-κB activation in primary AML cells is probably regulated at the same time by various cytokines and chemokines, considering that the addition of neutralizing antibodies of TNFα, IL-1 or IL-6 to AML cells did not influence the NF-κB activity [36, 76].

The crosstalk between AML and the bone microenvironment contributes to the disease progression, and it is plausible that paracrine-related cytokines also enhance the NF-κB activity in AML cells [77]. Recently, it has been shown that the interaction between bone marrow stromal cells and leukemic cells via binding of vascular cell adhesion molecule 1 (VCAM-1) to very late antigen 4 (VLA-4) induced NF-κB activation in the leukemic cells [78]. Besides this interaction, increased SPARC expression has also been observed in AML cells [79]. SPARC is a matricellular protein involved in the communication with the microenvironment [80]. It was shown that the expression is controlled by the SPI/NF-κB transactivation complex, and that secreted SPARC activated the integrin-linked kinase/AKT (ILK/AKT) pathway in an autocrine-dependent manner. This resulted in increased β-catenin activity, which regulated the self-renewal of AML cells. However, it has not been investigated whether SPARC contributes to the increased NF-κB activity in a positive feedback loop. Nevertheless, a combination of both autocrine and paracrine-related cytokines could most likely be required to induce and maintain constitutive NF-κB activation in AML cells.

2.3.3 INCREASED EXPRESSION OF NF-κB SIGNALING COMPONENTS INDUCES CONSTITUTIVE NF-κB SIGNALING

Besides this increased autocrine and paracrine signaling, increased expression and activation of multiple components of the NF-κB signaling pathway could potentially result in constitutive activation of NF-κB (Figure 2.3). Indeed, high activity of key components of the NF-κB pathway has been demonstrated in a wide variety of malignancies, hematological and otherwise [5]. In recent years, increased expression and activation of NF-κB signaling components has also been observed in AML cells.

Firstly, overexpression of the IL-IRAP has been found in stem and progenitors cells from AML and CML patients and has been correlated with the prognosis of AML and MDS patients [81–84]. Furthermore, in cord blood CD34+ cells it has been shown that IL-IRAP expression increased upon retroviral transduction of BCR-ABL [84]. Targeting the IL-IRAP protein in AML and CML cells by anti-IL-IRAP
antibodies induced antibody-dependent cell-mediated cytotoxicity, indicating that IL-IRAP is a promising target for the treatment of AML [84, 85].

Secondly, increased expression and activation of IRAK1, which can be activated by IL-1 and TLR-signaling, has been detected in high-risk MDS and AML cells [86]. Inhibition of IRAK1 by an IRAK inhibitor repressed NF-κB activation and suppressed MDS growth in vitro and in vivo. Moreover, simultaneous inhibition of IRAK and BCL-2 induced a synergistic cytotoxic effect, showing that IRAK1 could also be a potential target.

Thirdly, it has been suggested that inhibition of TAK1 could be a therapeutic option in the treatment of AML [87]. TAK1 is frequently overexpressed in primary AML cells, and genetic or chemical suppression of TAK1 induced apoptosis of AML cells in vitro and vivo. TAK1 inhibition suppressed multiple downstream pathways, including NF-κB, ERK, JNK and p38. Cell death mediated by TAK1 inhibition could at least partially be rescued by NF-κB overexpression, suggesting an important role of NF-κB downstream of TAK1 activity. However, upon TAK1-depletion multiple signaling pathways, including JNK, are suppressed, which might contribute to the
cytotoxic effect. In line with this hypothesis, it has been found that simultaneous inhibition of NF-κB and JNK has a synergistic effect in TNFα-expressing AML cells [88]. In addition, BTK which activates NF-κB downstream of TLR signaling is highly expressed and constitutively phosphorylated in AML [89].

Together, these findings suggest that components within the NF-κB signaling pathway contribute to the ongoing activation of NF-κB in AML cells. However, the molecular mechanism underlying the increased expression of most of these NF-κB signaling components in AML is still largely unknown. miRNAs might play an important role in the decreased expression of many of the NF-κB signaling components. Indeed, deletion or downregulation of NF-κB-related miRNAs has been found in various hematological malignancies [5, 90]. As mentioned previously, decreased expression of miRNAs could result in increased expression of TRAF6 and IKK. However, further studies are warranted to unravel the role of miRNAs on the expression of the different components within the NF-κB signaling pathway.

Regulators cooperating with the NF-κB complex in gene activation could potentially also play a role in the constitutive activation of NF-κB. BRD4 has been implicated as playing an important role in leukemia maintenance. Inhibition of BRD4 by using short hairpins significantly delayed MLL-AF9/NRASG2D leukemic outgrowth in mice [91]. Secondly, targeting of BRD4 by the chemical compounds JQ1 and IBET-151 induced apoptosis in primary leukemic cells and prolonged the survival of mice in MLL-AF4 and MLL-AF9 leukemia models [91, 92]. The effect of BRD4 inhibition on the survival of AML cells might be due partly to its association with NF-κB. Recently, it has been shown that constitutive activation of NF-κB in cancer cells is maintained by the binding of p65 to BRD4, thereby suppressing the ubiquitination of p65 and its degradation by the proteasome [19]. In addition, the effect of the BRD4 inhibitor IBET-151 is partially mediated via inhibition of BCL-2, a key target gene of NF-κB [92]. Moreover, TNFα addition and subsequent NF-κB activation resulted in the formation of new super enhancers. These super enhancers are hyperacetylated and have high levels of BRD4 at the expense of the basal super enhancers [93]. These data could indicate that leukemic cells that have gained constitutively activated NF-κB mediated by TNFα are more dependent on specific super enhancers. However, future studies are required to further illuminate the importance of NF-κB as an effector in BRD4-mediated gene transcription.

2.3.4 INCREASED PROTEASOMAL ACTIVITY IS RELATED TO CONSTITUTIVE NF-κB ACTIVITY

Protein homeostasis is essential for eukaryotic cells and is mediated by a tightly regulated synthesis and breakdown of proteins. It was recently reported that an increased or decreased protein synthesis rate largely impaired the function of HSCs, indicating that the synthesis rate of proteins is a highly controlled process in hematopoietic stem cells [94]. The degradation of proteins is on the other hand mediated via lysosomes and the ubiquitin-proteasome system. Whereas the lysosomal pathway is not selective, the ubiquitin-proteasomal pathway selectively degrades ubiquitinated proteins. The 26S proteasome complex has a barrel-like structure and is composed
of two outer α-rings and two central β-rings (20S), each formed by seven subunits and two regulatory caps (19S) that bind to ubiquitinated proteins. Proteolysis is mediated via the βi/PSMB6, β2/PSMB7 and the β5/PSMB5 subunits, which have respectively a caspase-like, trypsin-like and chymotrypsin-like activity. Due to its important role in the degradation of proteins, the proteasome is critically involved in many cellular processes, such as proliferation, apoptosis, DNA repair and possibly carcinogenesis [95]. Proteosomal activity is directly related to NF-κB activity via the degradation of ubiquitinated phosphorylated IκBα, as described above. Besides this constitutive proteasome, hematopoietic cells also contain an immunoproteasome variant, which has three catalytic subunits, β5i/PSMB8, βi/i/PSMB9 and β2i/PSMB10 [96, 97]. The immunoproteasome has been recognized as playing an important role in antigen presentation. Increased expression and activity of the proteasome has been observed in leukemic cells compared to normal hematopoietic cells [98, 99]. In addition, we have observed increased expression of 9 out of the 17 proteasome subunits in AML cells by comparing 66 AML CD34+ patient cells versus 22 NBM CD34+ cells by microarray [82, 83]. Furthermore, we also detected increased proteasome activity in AML CD34+ cells compared to NBM CD34+ cells. Increased proteasome activity has also been observed in leukemia-initiating cells (LICs) from murine MLL-ENL, MOZ-TIF2 or BCR-ABL/NUP98-HOXA9 models [38]. Importantly, it has been shown that these elevated levels of proteasome activity are related to increased NF-κB activity in a TNFα-dependent manner.

### 2.4 Targeted therapy of NF-κB signaling pathways

Unraveling the molecular mechanism of constitutive NF-κB activation is required to elucidate novel approaches to inhibit NF-κB activity in AML. As described above, various regulators that induce NF-κB activity, such as IRAK1, TAK1 and IKK, have been found to be potential targets for the treatment of AML (Table 2.2). However, proper validation in in vivo models using primary AML cells is needed to determine the functionality of these inhibitors on AML stem cells. In addition, further insight into the cooperation of NF-κB and BRD4 in gene transcription will be important to determine the extent to which the cytotoxic effects of the BRD4 inhibitors JQ1 and IBET-151 in AML are mediated via repression of NF-κB.
Besides these novel strategies, proteasomal inhibition has already been studied in more detail. Interestingly, the first compound that has been shown to inhibit NF-κB activity in AML cells is the proteasome inhibitor MG-132, which induced apoptosis in AML CD34+ cells while sparing normal CD34+ cells [37]. The first proteasome inhibitor that showed clinical effectiveness in AML patients was bortezomib, which primarily binds reversibly to the β5-subunit and blocks the chymotrypsin-like activity of the proteasome [104–106]. However, AML stem cells are largely insensitive to bortezomib, which might be due to improper reduction of NF-κB activity and upregulation of the anti-apoptotic protein MCL-1 in AML CD34+ cells [107]. Furthermore, neurotoxicity has been observed frequently, warranting the need for other proteasome inhibitors [108]. Recently, the second-generation proteasome inhibitors carfilzomib and its orally bio-available derivate oprozomib have been developed. These epoxyketone derivates bind irreversibly to the β5-subunit of the immuno(proteasome), which might increase the efficacy of these compounds by inhibiting the proteasome activity for a longer period [103]. Clinical activity of carfilzomib has been observed in bortezomib-refractory and bortezomib-relapsed multiple myeloma patients, and less severe side effects compared to bortezomib have been seen up to now [109–111]. In vitro experiments have shown that carfilzomib also induces apoptosis in AML blasts [112, 113]. Sensitivity of AML blasts is related to a high ratio of immunoproteasome levels compared to constitutive proteasome levels, which is likely due to the increased affinity of the second generation inhibitors to the immunoproteasome [97, 114]. Furthermore, we have recently shown that carfilzomib effectively induces apoptosis in primitive leukemic cells. In our study, the in vitro stem cell frequency was decreased upon carfilzomib treatment. This coincided with a reduction in quiescent CD34+CD38− cells. However, the clinical effectiveness of the second-generation proteasome inhibitors in AML patients, and accordingly in AML stem cells, has yet to be determined. It will also be important to evaluate the combination of proteasome inhibitors with MCL-1 inhibitors such as obatoclax, since the degradation of the anti-apoptotic protein MCL-1 is also dependent on the proteasome activity.

Inhibition of BTK could be another potential strategy for the treatment of AML. The irreversible BTK inhibitor ibrutinib has already been evaluated in phase 1 and 2 clinical studies, in which it showed cytotoxic effects in a variety of hematological malignancies, including CLL [115, 116]. In accordance with these results, ibrutinib induced cell death in primary AML cells, which coincided with decreased NF-κB, AKT and ERK activity [89]. BTK is also an important regulatory component of B cell receptor (BCR) signaling. However, it is currently unknown whether inhibition of BCR signaling in AML cells by ibrutinib plays a role in its cytotoxic effects.

As described above, overexpression of FLT3 or activating mutations of FLT3 triggered the NF-κB pathway [36, 63]. To further elucidate the mechanism-of-action of FLT3-inhibitors such as AC-220 [117], it would be interesting to study whether FLT3 inhibitors induce cytotoxicity in AML cells via repression of NF-κB.

Regarding the clinical application of these various inhibitors, it will be necessary to investigate the relationship between the cause of NF-κB activation and the clinical responsiveness of the inhibitors. For example, it has been observed
that TNFα-dependent growth of AML cells is not repressed by BTK inhibition. A possible explanation is that the repression of NF-κB signaling by BTK inhibition is compensated by constitutive activation of NF-κB via TNFα. This suggests that ibrutinib most likely has higher efficacy in AML cells expressing low levels of TNFα. In contrast, GM-CSF-, G-CSF- or IL-3-dependent growth was inhibited upon ibrutinib addition, as these cytokines do not activate NF-κB, or activate it to a lesser extent than TNFα [89, 118].

At present it is also unknown whether increased levels of BTK or IRAK1 directly correlate with the sensitivity of AML cells to ibrutinib or to IRAK1 inhibitors. Elucidating these correlations will be essential for the use of these inhibitors in the clinic.

In conclusion, understanding the various molecular mechanisms resulting in constitutive NF-κB activation has provided novel targets to inhibit NF-κB activity. Future research will be necessary to validate the importance of these targets. In addition, unraveling the exact role of the binding partners, such as BRD4 and CBP/p300 in NF-κB mediated transcription, might lead to the identification of novel strategies to inhibit NF-κB.
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