The TAK1-NF-κB axis as therapeutic target for AML

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Key Points

- The threonine kinase TAK1 is a survival-related gene that is strongly upregulated in AML CD34+ cells versus NBM CD34+ cells.
- Genetic and pharmacological inhibition of TAK1 induced cell death in a NF-κB dependent manner in AML cells in vitro as well as in vivo.
Abstract

Development and maintenance of leukemia can partially be attributed to alterations in (anti) apoptotic gene expression. Genome-wide transcriptome analyses revealed that 89 apoptosis-associated genes were differentially expressed between patient acute myeloid leukemia (AML) CD34+ cells and normal bone marrow (NBM) CD34+ cells. Amongst these, TGF-β activated kinase 1 (TAK1) was strongly upregulated in AML CD34+ cells. Genetic downmodulation or pharmacological inhibition of TAK1 activity strongly impaired primary AML cell survival and cobblestone formation in stromal co-cultures. TAK1 inhibition was mainly due to blockade of the NF-κB pathway, as TAK1 inhibition resulted in reduced levels of p-IκBα and p65 activity. Overexpression of a constitutive active variant of NF-κB partially rescued TAK1-depleted cells from apoptosis. Importantly, NBM CD34+ cells were less sensitive to TAK1 inhibition compared to AML CD34+ cells. Knockdown of TAK1 also severely impaired leukemia development in vivo and prolonged overall survival in a humanized xenograft mouse model. In conclusion, our results indicate that TAK1 is frequently overexpressed in AML CD34+ cells, and that TAK1 inhibition efficiently target leukemic stem/progenitor cells in a NF-κB-dependent manner.
Introduction

Due to a high incidence of relapse, the survival rate of AML patients is still below 30%, despite intensive treatment with chemotherapy. It is assumed that a small population of quiescent leukemic stem cells (LSCs) with self-renewal properties persists within the bone marrow microenvironment. These LSCs are responsible for relapse of the disease post-treatment\textsuperscript{1, 2}, suggesting that current therapies particularly target the more rapidly dividing leukemic blasts, while the LSCs generally survive.

Leukemogenesis of stem cells is a process in which various cellular programs can be affected, including those that regulate apoptosis and differentiation.\textsuperscript{3} Modification of programmed cell death might be important not only for leukemic transformation but could also contribute to tumor maintenance and chemoresistance. Dysregulation of a number of cell survival pathways, such as BCL2, p53, and NF-κB, have been associated with aberrations in apoptotic responses of AML cells.\textsuperscript{4} In contrast to normal bone marrow CD34\textsuperscript{+} cells, constitutive activation of NF-κB has been observed in AML CD34\textsuperscript{+} cells.\textsuperscript{5-8} In accordance with this activation, NF-κB-associated pathways are related to tumor formation and maintenance.\textsuperscript{9} Pharmacological inhibition of NF-κB by proteasome inhibitors has been reported to induce cell death in the AML CD34\textsuperscript{+}CD38\textsuperscript{−} subfraction both \textit{in vitro} and \textit{in vivo}.\textsuperscript{7, 8}

TGF-β activated kinase 1 (TAK1/MAP3K7) is a kinase upstream of NF-κB which can be activated by a variety of cytokines including TNFα, TGF-β and IL-1. Subsequently, phosphorylation of TAK1 leads to downstream activation of several pathways, including the NF-κB, JNK, ERK and p38-pathways.

Throughout embryonic development, TAK1 is required for angiogenesis and regulates the survival of endothelial cells and normal hematopoietic stem cells (HSCs).\textsuperscript{10, 11} A strong
reduction of HSCs in TAK\(^{-/-}\) mice has been observed that can partially be rescued by the knockout of tumor necrosis factor receptor1 and 2 (TNFR1 and TNFR2), suggesting that apoptosis upon TAK1 inhibition is largely mediated by TNF\(\alpha\) signaling.\(^{12,13}\) In various cancer models, including colorectal cancer, skin tumors and mantle cell lymphoma, TAK1 has been shown to be essential for the survival of cancer cells, where it might affect tumor metastasis in a WNT-dependent manner.\(^{14-18}\) Furthermore, reduction of TAK1 activity specifically induced cell death within all these different tumors, both \textit{in vitro} as well and \textit{in vivo}. Studies in patients with esophageal squamous cell carcinomas and clear cell renal cell carcinoma have also demonstrated that high TAK1 expression is associated with unfavorable prognosis.\(^{19,20}\)

We performed a detailed analysis of the apoptotic programs within the leukemic stem cell enriched CD34\(^+\) fraction of AML patients in comparison to normal CD34\(^+\) stem/progenitor cells. We discovered that TAK1 expression is elevated in a large subset of AML CD34\(^+\) cells. Pharmacological or genetic inhibition of TAK1 induced cell death in AML CD34\(^+\) cells in an NF-kB-dependent manner and significantly prolonged survival \textit{in vivo}. These results indicate that targeting of TAK1 could be a potential new strategy in the treatment of AML.
Methods

Micro-array analysis

Gene expression profiling apoptosis related genes was based on previously studies using Illumina HumanHT-12 Expression BeadChips. These studies included 100 samples and were divided as follows: 62 AML CD34+ (4 in duplicate), and 38 normal bone marrow CD34+ samples. All samples were corrected for background using Illumina GenomeStudio and then jointly forced to positive values, normalized and transformed using the R packages Bioconductor and Lumi. Probes with a detection p-value larger than 0.01 in all samples, as provided by GenomeStudio, were deleted. Log2 transformation and quantile normalization were applied. As a measure of quality control we performed a principal component analysis (PCA) on the correlation matrix of all 100 samples. The first component was removed from the data. To ensure reliability and reproducibility of the results we used multivariate permutations (MP) to determine the significance of our results.

A gene list composed of 386 apoptosis-related genes (in total 650 probes) was constructed. This list was largely based on Gene ontology terms 0097191 (extrinsic apoptotic signaling pathway) and 0097193 (intrinsic apoptotic signaling pathway) combined with human apoptotic proteins present in the uniprotKB database. Differential expression was considered significant at \( P < 0.0001 \). Average linkage hierarchical clustering with the centered correlation distance metric was performed using Cluster 3.0 and TreeView software (http://www.eisenlab.org/eisen/). Micro-array data have been deposited in GEO, accession number GSE30029.

Cell culture
The human promyelocytic leukemia cell line HL-60 and the human monocytic cell line MOLM13 were cultured in RPMI 1640 supplemented with 10% FBS. The human monocytic leukemia cell line Oci-AML3 was cultured in RPMI 1640 supplemented with 20% FBS. The human erythroleukemic cell line TF-1 was cultured in RPMI 1640 supplemented with 10% FCS and 10 ng/mL GM-CSF (Genetics Institute, Cambridge, MA).

**Primary AML cells, NBM and long term cultures on stroma**

Informed consent was obtained to use the AML blasts in accordance with the Declaration of Helsinki; the protocol was approved by the Medical Ethics Committee of the UMCG. AML mononuclear cells were isolated by density gradient centrifugation, and CD34+ cells were selected by MiniMacs (Miltenyi Biotec, Amsterdam, the Netherlands). Thereafter cells were expanded on MS5 stromal cells in long-term culture (LTC) medium (αMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% horse serum (Sigma, Zwijndrecht, the Netherlands), penicillin and streptomycin, 2 mM glutamine, 57.2 μM β-mercaptoethanol (Sigma) and 1 μM hydrocortisone (Sigma)) with interleukin 3 (IL-3; Gist-Brocades, Delft, the Netherlands), granulocyte colony-stimulating factor (G-CSF; Rhone-Poulenc Rorer, Amstelveen, the Netherlands), and thrombopoietin (TPO; Kirin, Tokyo, Japan) (20 ng/mL each) as previously described. Cultures were kept at 37°C and 5% CO2. Cultures were demipopulated weekly for analysis.

After achieving informed consent, bone marrow aspirates were obtained from patients who underwent a total hip replacement. The protocol for bone marrow collection was approved by the institutional review board of the University Medical Center Groningen (UMCG). All participants had normal general health, normal peripheral blood counts and did not suffer from a haematological disorder.
**Flow cytometry analysis**

Antibodies were obtained from Beckton Dickinson and Biolegend (Alphen a/d Rijn, the Netherlands). Cells were incubated with antibodies at 4°C for 30 minutes. All fluorescence-activated cell sorter (FACS) analyses were performed on a FACScalibur (Becton Dickinson) and data was analyzed using FlowJo 7.6.1. Cells were sorted on a MoFLo-XDP or Astrios (DakoCytomation, Carpinteria, CA, USA).

**Lentiviral and retroviral transductions**

ExtGLuc was retrieved from the rPLSI1180-ExtGLuc-IRES-hrGFP vector (kindly provided by the Brentjens lab) by EcoRI/StuI (blunt) and ligated into EcoRI/SalI (blunt) sites of the 3rd generation lentivector CD711B_1_pCDH_MSCV. Lentiviral particles were produced by transient transfection in 293T cells using pVSV, pREV and pMDL-PRRE helper plasmids. The lentivirus short hairpin RNA vectors targeting human TAK1 were obtained from Open Biosystems (Thermo Scientific, Clone ID: TRCN0000001554, TRCN0000001555, TRCN0000001556 and TRCN0000001558) targeting the following sequences: (1) AAACAATCCAAGAATCACTGC, (2) TATTAGGATGGTTCACACGGG, (3) ATTCCATCACAAGACACACTG, and (4) ATAGTATCATTGTTGGCAGGA. These hairpins were cloned into the pLKO.1 lentiviral vector containing GFP (kindly provided by Dr. J. Larsson) or mCherry. The pLKO.1 GFP vector containing a scrambled (SCR) short hairpin was used as control vector. Lentiviral particles were produced by transient transfection of 293T cells with the lentiviral expression vectors, and stable transduction of AML cell lines or CD34+ AML cells was performed, which have both been extensively described. The retroviral construct pCMV IKKβ S177E S181E has been described previously. Retroviral
particles were made by transient transfection of PG13, and OCI-M3 cells were transduced with these particles. Transduction efficiency was measured by FACS analysis. Knockdown was investigated by quantitative reverse-transcription–polymerase chain reaction and Western blot.

**NF-κB assay**

NF-κB activity was measured by ELISA. ELISA was performed using the TRANS-AM NF-κB p65 Transcription Factor Assay Kit (Active Motif, North America, Carlsbad, CA) following the manufacturer’s recommendations and as described previously.31

**NF-κB luciferase reporter assay**

293T cells were co-transfected with a luciferase vector containing three NF-κB responsive elements (NRE)32 together with the MIGR1 or IKK SSEE vectors. Luciferase assays were performed as described previously.30

**Migration assay**

Migration assay of MOLM13 cells was performed in a transwell system (Corning Costar, Cambridge, UK) with 8 μm pore size. Two days after transduction, cells were resuspended in 100 μL medium and added to the upper chamber; 600 uL of medium with and without 100 ng/mL SDF-1 was added to the lower chamber. Cells were incubated for 4 hours at 37°C and migrated and non-migrated cells were counted.

**Animal experiments**

Eight to ten week old female NSG (NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ) were purchased from the CDP breeding facility within the UMCG. Mouse experiments were performed in
accordance with national and institutional guidelines, and all experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG). Prior to transplantations, mice were sub-lethally irradiated with a dose of 1.0 Gy. Following irradiation mice received Neomycin (3.5 g/l in drinking water) and soft food daily for 2 weeks. Mice were lateral tail vein injected with 200,000 MOLM13 luciferase GFP\(^+\) transduced with SCR or shTAK1 mCherry lentivirus.

Bioluminescence imaging was performed 17 days after injection of the cells using the IVIS Spectrum, whereby 100 \(\mu\)g of coelenterazine was intravenously injected into the retro-orbital plexus and the mice were imaged for 30 seconds. Bioluminescence was quantified using Living Image 2.50. Mice were sacrificed upon detection of clinical signs of disease.

Supplemental Methods are available online.
Results

Transcriptome profiling identified differences in apoptotic signaling in AML CD34+ cells compared to normal bone marrow CD34+ cells

To evaluate the apoptotic programming in LSC-enriched AML CD34+ cells, transcriptome analyses was performed on a previously established gene expression data set of a large cohort of patient AML CD34+ cells (n=62) and compared to NBM CD34+ cells (n=38). Gene set enrichment analysis (GSEA) was performed, which revealed multiple apoptosis-associated sets to be highly enriched in AML CD34+ cells. In the top 30 significantly enriched gene sets, six sets were related to apoptosis, suggesting a major change in apoptotic programming in AML CD34+ cells compared to normal CD34+ cells (Figure 1A). Next, we determined the apoptosis-associated genes that were differentially expressed between AML and normal CD34+ cells by performing transcriptome analysis on 386 apoptosis-associated genes (Suppl. Table 2). In total, 89 genes were differentially expressed between AML and normal CD34+ cells (p < 1e-6). Supervised cluster analysis using these 89 genes, revealed that AML CD34+ cells and NBM CD34+ cells indeed clustered into two groups (Suppl. Figure 1). Interestingly, the expression of various anti-apoptotic as well as pro-apoptotic genes was increased in AML CD34+ cells compared to NBM CD34+ (Suppl. Table 3). A heatmap of the 25 most significantly upregulated genes is shown in Figure 1B, where the survival-related gene TAK1 was significantly higher expressed in AML CD34+ cells compared to NBM CD34+ cells. TAK1 was previously identified as one of the components of the leukemic stem cell-related gene profile which were significantly higher expressed in the AML LSC compared to the non-LSC fraction. Gene array data for TAK1 expression in all individual samples is shown in Figure 1C, showing an increased expression of TAK1 in a large subset of AML CD34+ cells. Elevated expression of TAK1 was independently confirmed by Q-PCR (Figure 1D) and
Western blotting (Figure 1E). These findings indicate that TAK1 is overexpressed in AML CD34+ cells.

**Inhibition of TAK1 impaired expansion of AML cell lines by increased apoptosis.**

To study the functional consequences of TAK1 expression in AML cells, AML cell lines were exposed to the TAK1 inhibitors 5z-7-oxozeaenol and AZ-TAK1, which block the phosphorylation and thereby activity of TAK1.\(^{34}\) Whereas 5z-7-oxozeaenol is a resocyclic acid lactone of fungal origin, AZ-TAK1 has recently been discovered in a small molecule-based lead identification to find compounds that specifically inhibit TAK1 phosphorylation. Both compounds inhibit the kinase activity at a nanomolar range (8 nM) by binding into the ATP binding pocket.\(^{14,34,35}\) In the present study we tested the functionality of these inhibitors on the AML cell lines HL60, OCI-M3 and MOLM13. Low concentrations of 5z-7-oxozeaenol and AZ-TAK1 resulted in increased apoptosis in most of the cell lines (Figure 2A) and could be promoted further when 5z-7-oxozeaenol was combined with TNF\(\alpha\), as previously indicated.\(^{36}\) With the AZ-TAK1 inhibitor, comparable effects were observed with or without TNF addition; therefore the additional experiments with AZ-TAK1 were performed without TNF. To confirm the specificity of the effects, we constructed two independent pLKO.1 GFP short hairpins targeting TAK1. Efficient downmodulation of TAK1 in HL60 cells was confirmed at the RNA and protein level (Figure 2B and 2C). TAK1 depletion resulted in a growth disadvantage of AML cell lines (Figure 2D). This growth disadvantage was due to enhanced apoptosis as indicated by an increase of Annexin V positive cells over time and the presence of cleaved caspase 8 (Figure 2E-2F). Taken together, these results indicate that TAK1 is critical for the cell survival of AML cell lines.
Cell death induced by TAK1 inhibition was clearly associated with inhibition of the NF-κB pathway

To unravel the downstream signaling pathways responsible for the TAK1 mediated cell death, various known pathways downstream of TAK1 were studied. Upon inhibition of TAK1 by 5z-7-oxozeaenol, phosphorylation of JNK, p38, ERK and IκBα was inhibited (Figure 3A). Downstream activation of NF-κB was completely abolished, as demonstrated by a p65 activity assay (Figure 3B). Furthermore, a decrease in IL8 mRNA levels, which transcription is NF-κB dependent, was observed upon addition of 5z-7-oxozeaenol or transduction with TAK1 hairpins (Suppl. Figure 2A/B). To determine which downstream pathways were mainly responsible for TAK1 mediated cell death, AML cell lines were treated with either the JNK inhibitor SP600125, MEK/ERK inhibitor U0126, the NF-κB inhibitor BMS-345541 and p38 inhibitor SB203580, alone or in combination with TNFα. Addition of the NF-κB inhibitor BMS-345541 induced apoptosis in MOLM13, OCI-M3 and HL60 cells, which increased significantly in combination with TNFα (2.4 fold, p = 0.02) (Figure 3C). Interestingly, the sensitivity of the three AML cell lines to the NF-κB inhibitor was comparable to the sensitivity to both TAK1 inhibitors. In contrast, inhibition of the p38, MEK/ERK and JNK signaling pathways, either alone or in combination with TNFα, caused limited cell death (<14%) (Figure 3C) although the p38 inhibitor, MEK/ERK inhibitor and the JNK inhibitor were effectively inhibiting their target (Suppl. Figure 2C). To show that TAK1-mediated survival was mainly due to decreased NF-κB activation, we transduced the OCI-M3 cells with IKKβ S177E, S181E (IKK SSEE) mutant which mimics the active state of p65.37 Constitutive activation of NF-κB by these construct was detected by a performing a NF-κB luciferase reporter assay. This assay confirmed that expression of IKK SSEE constitutively triggered NF-κB activation in these cells (Suppl. Figure 3). The cell death induced by the TAK1 inhibitor AZ-TAK1 was significantly reduced (p=0.03) upon overexpression of IKK SSEE.
OCI-AML3 cells, indicating that NF-kB is an important mediator of the pro-survival pathway downstream of TAK1 (Figure 3D).

c-FLIP<sub>L</sub> is one of the anti-apoptotic gene targets of NF-κB and has also been shown to overcome the cell death-inducing effect of TAK1 in MEFs.<sup>38</sup> Therefore to determine whether a correlation exists between TAK1 and cFLIP<sub>L</sub>, RNA levels of c-FLIP<sub>L</sub> were measured in shTAK1 transduced HL60 cells. Downmodulation of TAK1 resulted in reduced gene expression levels of cFLIP<sub>L</sub> (relative expression level is 40% ± 14%, p < 0.05) (Figure 3E), indicating that c-FLIP<sub>L</sub> is one of the pro-survival signals provided by the TAK1-NF-κB axis.

Whereas NF-kB is commonly constitutively activated in AML<sup>5,7</sup>, we verified whether AML CD34<sup>+</sup> cells expressing high levels of TAK1 also have increased NF-κB activity by determining IL8 levels. We observed that AML CD34<sup>+</sup> cells indeed express increased levels of IL8 (Suppl. Figure 2D). Nevertheless, TAK1 levels were not linearly correlated with IL8 levels (Suppl. Figure 2E and 2F) and a number of AMLs that expressed low levels of TAK1 also expressed high levels of IL8. This is possibly due to enhanced NF-κB activity that is observed in a wide spectrum of leukemia subtypes which might reflect abnormalities or mutations of activators of NF-κB which activate NF-κB independent of TAK1, such as RAS<sup>5</sup>.

**Impaired long-term growth of AML CD34<sup>+</sup> cells on bone marrow stroma after inhibition of TAK1**

To assess whether TAK1 inhibition also affects the survival of primary AML CD34<sup>+</sup> cells, we investigated the effectiveness of the TAK1 inhibitor AZ-TAK1 on primary AML CD34<sup>+</sup> cells. After 24 hrs of incubation with AZ-TAK1 of AML CD34<sup>+</sup> cells, we observed a concentration-dependent effect on cell death (Figure 4A) which equally affected the AML CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>-</sup> cell fraction (data not shown). In line with the reduction of NF-κB activity in AML cell lines upon TAK1 inhibition, a decrease in NF-κB activity was also seen in AML
CD34+ patient cells after addition of 100 nM and 500 nM AZ-TAK1 (n=3) (Figure 4B). More importantly, long-term growth of primary AML CD34+ cells (n=5) on MS5 stromal layer was reduced upon addition of 100 nM AZ-TAK1 (Figure 4C). Moreover, addition of 500nM AZ-TAK1 completely abrogated the out-growth of AML CD34+ cells and no cobblestone formation could be observed under the MS5 stromal layer (Suppl. Figure 4).

To verify the specificity of the effects, AML CD34+ cells (n=3) were transduced with both TAK1 hairpins, and after 2 days GFP+ cells were cultured on MS5 stromal layer. A significant reduction in growth of TAK1-hairpin transduced AML cells was observed in time, whereas scrambled transduced cell were able to expand on the stromal cells (Figure 4D). Also, a reduction of cobblestone frequency was observed in the shTAK1 AML cell cultures. Loss of suspension and adhesion cells in these long-term assays indicated that the primitive leukemic progenitor and stem cells were targeted \textit{in vitro}.

**Normal bone marrow cells less affected upon TAK1 inhibition**

To evaluate the effect of TAK1 inhibition on the survival of normal hematopoietic cells, NBM CD34+ cells (n=5) were incubated with 100 nM AZ-TAK1 for 24 hrs and thereafter plated in methylcellulose. TAK1 inhibition resulted in a 2 fold decrease in CFC numbers (Figure 5A). In line with this data, shTAK1 transduced cord blood CD34+ cells also resulted in a 2 fold reduction in CFC numbers when plated in methylcellulose (Figure 5B).

Interestingly, upon treatment with 100 nM AZ-TAK1 for 24 hrs, a small increase in Annexin V+ cells was observed in NBM CD34+ cells (relative survival is 77% +/- 9%, n=6) upon TAK1 inhibition. However, the suppressive effect was significantly less pronounced (p < 0.01) compared to AML CD34+ cells (n=8) treated for 24 hrs with 100 nM AZ-TAK1 (Figure 5C). The observed variability in sensitivity between the various AML CD34+ cells was not related to expression levels of TAK1.
TAK1 inhibition resulted in impaired leukemia development and prolonged survival in a MOLM13 xenograft model.

Since the bone marrow microenvironment might provide important signals that attenuate the effects seen upon TAK1 inhibition, we verified the effect of TAK1 inhibition on AML cells in vivo. MOLM13 Gaussia luciferase GFP positive cells were transduced with lentiviral TAK1 mCherry hairpin or control constructs (Figure 6A). Two days after transduction, cells were sorted and intravenously injected into NSG mice (Figure 6B). The effectiveness of knockdown of TAK1 mCherry hairpins was verified by Q-PCR (Suppl. Figure 5A). Two weeks after injection, chimerism levels in peripheral blood were significantly lower in shTAK1 mice compared to SCR mice (Figure 6C). Moreover, luciferase activity was detectable in all SCR mice within the bone marrow, liver and lungs, whereas shTAK1 mice only had minimal bioluminescence activity (Figure 6D and 6E). Kaplan Meier curves indicated that SCR mice survived only for 3 weeks (Figure 6F), with leukemia penetrance in bone marrow, blood, spleen and liver (Suppl. Figure 5B and 5C). In contrast, TAK1 knockdown resulted in a significantly improved survival (p < 0.01) (Figure 6F). Efficiency of TAK1 knockdown at day of sacrifice of some of the shTAK1 mice was lower than at the day of injection, suggesting that leukemia developed due to improper knockdown in these cells (Suppl. Figure 5D). In the 40% of the shTAK1 mice that did survive throughout the experiment, no leukemic cells could be detected in blood, bone marrow, liver and spleen at the time these mice were sacrificed. An impaired homing of the transduced cells is unlikely responsible for the observed findings since MOLM 13 cells transduced with shTAK1 had a comparable CXCR4 expression level as the control cells (Suppl. Figure 6A). In addition no difference in migration to SDF1 was observed between both cell populations. (Suppl. Figure
6B). These data indicate that targeting TAK1 strongly impairs leukemia development \textit{in vivo} and results in a significantly improved survival.
**Discussion**

Evasion of apoptosis is one of the hallmarks during the development of cancer. Overexpression of multiple anti-apoptotic genes of the mitochondrial related BCL-2 family has been related to the development and or maintenance of AML and is of prognostic significance for the treatment outcome of this patients group. By comparing the apoptotic gene profile of AML CD34+ cells versus normal CD34+ cells, we observed a high expression of several anti-apoptotic genes and, surprisingly, also some pro-apoptotic genes (Suppl. Table 2). This has previously been observed in an independent cohort of AML and chronic myeloid leukemia patients. It is assumed that the increased expression of anti-apoptotic genes, leading to oncogene addiction, is counterbalanced by elevated expression of pro-apoptotic genes, thus creating a “primed to cell death” status. In our present study, we observed increased expression of TAK1 in a large panel of AML CD34+ cells at RNA and protein level. Notably, TAK1 expression was recently shown to be part of a gene expression signature that defines AML stem cells. These findings led us hypothesize that TAK1 might play an important role in AML stem cell maintenance. The results of the present study indicate that TAK1 is highly relevant for the *in vitro* and *in vivo* survival of AML cells. TAK1 inhibition by genetic or pharmacological inhibition strongly triggered cell death in AML CD34+ cells.

We observed that the pro-survival function of TAK1 was largely dependent on NF-kB activity, and less dependent on MEK, p38 and JNK activity, although all these pathways are triggered by TAK1 activation. NF-κB inhibition not only phenocopied the effects observed with the TAK1 inhibitors, but cell death upon TAK1 inhibition could also be partially rescued by overexpression of NF-κB. It has been demonstrated that NF-κB activity is elevated in patient AML blasts and AML CD34+ cells compared to normal CD34+ cells, in part due to the autocrine and paracrine production of growth factors. So far it appears that the enhanced NF-κB expression in AML is a more general result of the malignant transformation and is not
related to specific AML subtypes or genetic abnormalities. It was recently demonstrated that NF-κB is activated in several leukemia mouse model systems including MLL-ENL and MOZ-TIF2, and in the double hit model of BCR-ABL with NUP98-HOXA9. Moreover, in these studies the constitutive activation of NF-kB affected the primitive leukemic stem cell fraction by expanding the numbers of leukemic initiating cells.\textsuperscript{51,52}

An important mediator for the constitutive activation of NF-κB is the autocrine production of TNFα by leukemic cells.\textsuperscript{53} Apparently, the transforming events triggered by various mutated genes favor pathways with a pro-survival signature, which are subsequently highly relevant for tumor maintenance. An alternative pathway for NF-kB activation might be the TRAF6 and Interleukin Receptor Associated Kinase-1 (IRAK1), which are interacting proteins and mediators of Toll-like (TLR) and Interleukin-1 (IL-1) receptors. Activation of TLR or IL-1R results in phosphorylation of IRAK1, leading to binding and activation of TRAF6. Thereafter, TAK1 will be phosphorylated, resulting in the subsequent activation of NF-κB. Recently, it was shown that IRAK1 is overexpressed and highly activated in high-risk MDS and AML.\textsuperscript{54} Inhibition of IRAK1 exhibited an impaired expansion and apoptosis and the co-treatment with BCL2 inhibitors eliminated the MDS clones.

Multiple transcriptome data sets of AML and normal CD34\textsuperscript{+} cells, including ours, indicate that besides TAK1, TRAF6 and IL1RAP are also significantly overexpressed in AML CD34\textsuperscript{+} cells\textsuperscript{22,55-57}, suggesting an ongoing activation of these pathways, subsequently leading to pro-survival signals. The higher expression of TAK1 in AML CD34\textsuperscript{+} cells compared to normal CD34\textsuperscript{+} cells suggests that leukemic cells are more dependent on this pathway than normal CD34\textsuperscript{+} cells for their survival, indicating a potential therapeutic window for drug targeting. The \textit{in vitro} results of the TAK1 inhibitor are in line with these data, showing a difference in susceptibility for leukemic versus normal cells. Apparently, this type of NF-kB inhibition is more effective than NF-κB inhibition by proteasome inhibition.\textsuperscript{31,58}
However, it cannot be excluded that other pathways besides NF-κB are affected, since NF-κB overexpression could only partially rescue the phenotype. In addition, knockout of the components of the NF-κB complex, p65/RelA and p52/RelB, results in a significant decline in HSCs frequency \(^{59, 60}\), but the phenotypes appear to be less severe in comparison to the TAK1 knockout mice.\(^{11}\) These results are in line with the findings that different pathways converge at TAK1 and that various pathways, including NF-κB, are activated downstream of TAK1. In summary, our data demonstrate that TAK1 is a critical component in leukemic stem cell maintenance and might be an innovative target for patient treatment.
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Author Contributions

Conceived and designed the experiments: MCJ, WJQ, JJS and EV. Performed the experiments: MCJ, HS, JJ, AZBV and JJS. Analyzed the data: MCJ, JJS and EV. Wrote the paper: MCJ, JJS and EV.
Conflict of Interest Disclosures

The authors have declared that no competing interests exist.
Reference List


Figure legends

**Figure 1: TAK1 expression is increased in AML CD34+ cell versus normal bone marrow CD34+ cells.**
A) Gene set enrichment analysis (GSEA) profiles generated from the AML CD34+ versus NBM CD34+ gene list. B) 25 most significantly upregulated apoptosis-associated genes in AML CD34+ cells versus NBM CD34+ cells C) Relative expression of TAK1 in all individual AML CD34+ and NBM CD34+ samples analyzed by gene profiling. D) Relative expression of TAK1 AML CD34+ and NBM CD34+ cells by using qPCR. E) Relative protein levels of TAK1 in 2 AML CD34+ cells expressing high RNA levels of TAK1 and 2 AML CD34+ cells expressing low RNA levels of TAK1.

**Figure 2: TAK1 inhibition induces apoptosis in AML cells**
A) HL60, OCI-M3 and MOLM13 cells were incubated with various concentrations of TAK1 inhibitors 5z-7-oxozeaenol and AZ-TAK1, alone or in combination with TNFα, and apoptosis was quantified by using Annexin V staining. B) Relative expression of TAK1 in HL60 cells using two independent hairpins targeting TAK1. D) Relative TAK1 protein expression of shTAK1 or scrambled transduced HL60 cells. E) Cell growth of MOLM13 cells transduced with TAK1 hairpins or scrambled hairpin. F) Annexin V staining of the shTAK1 or scrambled transduced MOLM13 cells G) Cleaved caspase-8 protein expression of shTAK1 and scrambled transduced HL60 cells at day 1 after transduction.
Figure 3: Apoptosis induced by TAK1 inhibition is mainly dependent on NF-kB pathway.

A) OCI-M3 cells were treated with various concentrations of 5z-7-oxozaenol, and western blot analysis was performed on phospho-IkBα, total IkBα, phospho-ERK, total ERK, phospho-c-JUN, total C-JUN, phospho-p38 and total p38. B) NF-κB activity of OCI-M3 cells treated with 100 nM 5z-7-oxozaenol. NF-κB activity was measured by p65 DNA binding ELISA. The negative control is the background (no nuclear extract). C) MOLM13, OCI-M3 and HL60 cells were treated with 10 μM JNK inhibitor SP600125, 5 μM MEK/ERK inhibitor U0126, 5 μM NF-κB inhibitor BMS-345541 or 1 μM p38 inhibitor SB203580, alone or in combination with TNFα. After 24 hrs of incubation, apoptosis was quantified by Annexin V staining. D) OCI-M3 cells were transduced with control MIGR1 or IKK SSEE vector and incubated with 80 nM AZ-TAK1. After 24 hrs, Annexin V+ cells were measured. E) Relative cFLIP₁ levels of SCR HL60 and shTAK1 cells quantified by qPCR.

Figure 4: Targeting of TAK1 impairs expansion of primary AML CD34+ cells

A) Primary AML CD34+ cells were incubated with various concentrations of AZ-TAK1 for 24 hrs and cell death was quantified by Annexin V staining. B) NF-κB activity of primary AML CD34+ cells treated with 100 nM AZ-TAK1. NF-κB activity was measured by p65 DNA binding ELISA. C) Growth of primary AML CD34+ cells treated with 100 nM AZ-TAK1. AZ-TAK1 was added after initial growth was observed and added at the indicated time points (↓). D) Growth of primary AML CD34+ cells transduced with hairpins targeting TAK1 or control hairpins
Figure 5: Difference in sensitivity to TAK1 inhibition between normal bone marrow CD34+ cells versus AML CD34+ cells
A) NBM CD34+ cells (n=5) were incubated with 100nM AZ-TAK1 for 24 hrs and after 2 weeks CFCs were determined. B) Cord blood CD34+ cells were transduced with hairpins targeting TAK1; after two weeks CFCs were determined. C) AML CD34+ cells (n=8) and NBM CD34+ cells (n=6) were incubated with 100 nM AZ-TAK1; after 24 hrs cell death was quantified by Annexin V staining.

Figure 6: TAK1 inhibition results in impaired leukemia development and increased survival in a MOLM13 xenograft model.
A) Schematic representation of mouse experiment. B) Sorting strategy of MOLM13 luciferase GFP positive cells transduced with either control mCherry vector or shTAK1 mCherry hairpin. C) Chimerism levels in peripheral blood after two weeks of injection of MOLM13 luciferase GFP/SCR mCherry cells or MOLM13 luciferase GFP/shTAK1 mCherry cells. D) Bioluminescence pictures of SCR mice and shTAK1 mice. E) Quantification of bioluminescence assay of SCR mice (n=6) and shTAK1 mouse (n=10). F) Survival curve of SCR mice and shTAK1 mice.
Figure 5

A

CFC/10^3 cells

control

100 nM AZ-TAK1

NBM CD34^+  

1 2 3 4 5

B

relative CFC frequency

control

AZ-TAK1 (100 nM)

SCR

sh1

sh2

C

SSC

AML CD34^+ (nr.11)

% survival (relative to control)

AML CD34^+

NBM CD34^+

AnnexinV-FITC
The TAK1-NF-κB axis as therapeutic target for AML

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