Positive feedback between NF-κB and TNF-α promotes leukemia-initiating cell capacity

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy that originates from leukemia-initiating cells (LICs). The identification of common mechanisms underlying LIC development will be important in establishing broadly effective therapeutics for AML. Constitutive NF-κB pathway activation has been reported in different types of AML; however, the mechanism of NF-κB activation and its importance in leukemia progression are poorly understood. Here, we analyzed myeloid leukemia mouse models to assess NF-κB activity in AML LICs. We found that LICs, but not normal hematopoietic stem cells or non-LIC fractions within leukemia cells, exhibited constitutive NF-κB activity. This activity was maintained through autocrine TNF-α secretion, which formed an NF-κB/TNF-α positive feedback loop. LICs had increased levels of active proteasomal machinery, which promoted the degradation of IkBα and further supported NF-κB activity. Pharmacological inhibition of the proteasome complex markedly suppressed leukemia progression in vivo. Conversely, enhanced activation of NF-κB signaling expanded LIC frequency within leukemia cell populations. We also demonstrated a strong correlation between NF-κB activity and TNF-α secretion in human AML samples. Our findings indicate that NF-κB/TNF-α signaling in LICs contributes to leukemia progression and provide a widely applicable approach for targeting LICs.

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different types of myeloid leukemia, we used three types of mouse models of myeloid leukemia induced by the retroviral transduction of granulocyte-monocyte progenitors (GMPs) with MLL-ENL and MOZ-TIF2 and the cotransduction of GMPs with BCR-ABL and NUP98-HOXA9 (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI68101DS1). LIC-enriched populations of these myeloid leukemia models have been investigated in previous studies: GMP-like leukemia cells (L-GMPs) in MLL-ENL and MOZ-TIF2 models and the lineage− Sca-1+ fraction in the BCR-ABL/NUP98-HOXA9 model (Supplemental Figure 2, A–C, and refs. 25–27). In order to obtain and lineage+ cells in a BCR-ABL/NUP98-HOXA9 model. There

B target genes were assessed by gene set enrichment analysis L-GMPs (28) with those of normal hematopoietic stem or progenitor cells (HSPCs) and GMPs (26, 28, 30). After narrowing down our analysis to the genes commonly upregulated in LICs in three different types of murine leukemia models, we further selected nineteen genes whose expression is elevated in human AML CD34+CD38− cells (Figure 3A). Among the nineteen genes with typically elevated expression levels in LICs, we focused on Tnf, because it is well known as an activator of NF-κB and as an NF-κB-regulated gene. For the purpose of directly evaluating TNF-α abundance in the BM of leukemic mice, we measured the concentration of TNF-α in the BM extracellular fluid and confirmed that it was conspicuously enriched in leukemic BM cells compared with normal BM cells (Figure 3B). We also examined the TNF-α concentration in culture media conditioned by LICs, non-LICs, and normal cells, respectively, to determine whether leukemia cells themselves have the ability to secrete TNF-α. We found that TNF-α secretion was distinctly elevated in LICs, while the normal GMP-conditioned media barely included TNF-α (Figure 3C). Although non-LICs also had TNF-α secretory ability, it was much lower than that of LICs. We therefore reasoned that LICs might maintain their NF-κB pathway activity via autocrine TNF-α signaling. To test this hypothesis, we cultured freshly isolated LICs in serum-free media with a TNF-α-neutralizing antibody or its isotype control and observed p65 nuclear translocation. While LICs treated with isotype control antibodies maintained p65 nuclear translocation even after serum-deprived culture, the p65 translocation signal we observed in three types of LICs was significantly attenuated when these cells were cultured with neutralizing antibodies against TNF-α (Figure 3D). The results were also confirmed by quantification of p65 intensity (Figure 3E). These data strongly suggest that different types of LICs have a similarly increased potential for TNF-α secretion, which maintains constitutive NF-κB activity in an autonomous manner.

Autocrine TNF-α signaling promotes leukemia cell progression. We were then interested in exploring the effect of autocrine TNF-α secretion on leukemia progression. BM cells derived from WT or Tnf−− knockout mice were transplanted into sublethally irradiated WT recipient mice after transduction with MLL-ENL and MOZ-TIF2, and cotransduction with BCR-ABL and NUP98-HOXA9 (Figure 3F). Although several mice did develop leukemia with prolonged latency, Tnf-deficient cells were significantly (p < 0.01) impaired in their ability to initiate leukemia (Figure 3G). We confirmed that Tnf−− LICs show a distinct decrease in nuclear localization of p65 compared with the that in LICs derived from WT BM cells (Supplemental Figure 5, A and B). Next, we examined whether paracrine TNF-α from the BM microenvironment contributes to leukemia progression. When the established leukemia cells were secondarily transplanted into WT or Tnf−− knockout recipient mice, Tnf−− leukemia cells failed to effectively establish AML in the Journal of Clinical Investigation http://www.jci.org Volume 124 Number 2 February 2014 529
Figure 1
NF-κB pathway is activated in LICs of different murine myeloid leukemia models. (A) LIC frequency in the two fractions of each leukemia model as determined by limiting dilution assay. See Supplemental Table 1 for detailed transplantation results. (B) Immunofluorescence assessment for p65 nuclear translocation in KSLs, GMPs, LICs, and non-LICs in three leukemia models. Scale bars: 10 μm. (C) Quantification of p65 nuclear translocation assessed by the mean nucleus/cytoplasm intensity ratio. More than 50 cells were scored in each specimen, and the average intensity ratio with SD is shown.
all three models (Figure 3, H and I). Interestingly, there was no significant difference in leukemogenicity among the recipient genotypes. These results indicate that autocrine TNF-α secretion is important for AML progression and that the contribution of paracrine effects derived from stromal cells is minimal.

**Figure 2**
NF-κB transcription activity is increased in LICs. (A) GSEA of NF-κB target genes in the published gene expression data comparing LICs in leukemia mouse models with normal HSPCs. Left panel: comparison of MOZ-TIF2 L-GMP with normal KSLs and GMPs (GSE24797). Right panel: comparison of MLL-AF9 and HOXA9-MEIS1 L-GMPs with normal KSLs, common myeloid progenitors (CMPs), and GMPs (GSE20377). (B) GSEA of NF-κB target genes in CD34⁺CD38⁻ fractions in human AML versus healthy controls (GSE24006). (C) Quantitative real-time PCR analysis of a subset of NF-κB target genes in LICs of MLL-ENL, MOZ-TIF2, and BCR-ABL/NUP98-HOXA9 leukemia models relative to normal GMPs (n = 4). Error bars indicate SD. (D) Immunoblotting of total and phosphorylated p65 in normal GMPs and LICs in the three leukemia models. (E) Representative annexin V and 7-AAD profiles of normal c-Kit⁺ cells, L-GMPs, and Lin⁻c-Kit⁻ cells in MLL-ENL leukemic mice after a 24-hour culture with or without 10 μM IKK inhibitor (sc-514). (F) Average percentage increase in apoptotic cells in LICs of the three leukemia models compared with that in non-LICs and normal c-Kit⁺ cells treated with 10 μM IKK inhibitor (sc-514) (n = 4 each). Error bars indicate SD.
with a control vector, transplanted them into recipient mice, and compared the characteristics of the repopulating cells (Figure 4A). Although the introduction of IκB-SR did not affect the morphology of MLL-ENL leukemia cells (Supplemental Figure 6A), p65 was almost completely sequestered in the cytoplasm of L-GMPs with IκB-SR (Figure 4B and Supplemental Figure 6B), and the expression levels of NF-κB target genes, including Tnf, were substantially decreased (Figure 4C). Considering that the blockage of autocrine TNF-α attenuated NF-κB signaling, we hypothesized that NF-κB activity and TNF-α secretion form a positive feedback loop in LICs. We therefore established MOZ-TIF2 and BCR-ABL plus NUP98-HOXA9 and transplanted into sublethally irradiated mice. (G) Survival curves of mice in the experiments shown in F (n = 7 each). (H) Schematic representation of the experiments. WT or Tnf−/− leukemia cells were secondarily transplanted into WT or Tnf−/− recipient mice. (I) Survival curves of mice in the experiments shown in H (n = 5 each).

BM cells. Then, the developed leukemia cells were infected with codon-improved Cre recombinase-IRESCFP (iCre-IRESCFP) or GFP empty vector, and GFP-positive cells were isolated and secondarily transplanted into sublethally irradiated mice (Figure 4F). Remarkably, most of the mice transplanted with Rela-deleted leukemia cells did not develop leukemia (Figure 4G). Compared with controls, several mice did develop leukemia after longer latencies, but they did not develop leukemia after tertiary transplantation (data not shown), indicating that the complete ablation of NF-κB drastically reduced leukemogenicity.

High proteasome activity in LICs yields differences in NF-κB activity between leukemia cell populations. We next sought to elucidate the mechanisms underlying the differences in p65 nuclear translocation status between LICs and non-LICs. We confirmed that LICs had substantially lower IκBα protein levels compared with those of non-LICs in all three models (Figure 5, A and B). These results are very consistent with the p65 distribution status of LICs and non-LICs, considering that NF-κB is usually sequestered in the cytoplasm, bound to IκBα, and translocates to the nucleus, where IκBα is phosphorylated and degraded upon stimulation with a variety of agents such as TNF-α (33). We initially tested whether the expression of IκBα is downregulated in LICs at the transcription level and found that LICs had a tendency toward increased Nfkbia mRNA expression levels compared with non-LICs (Figure 5C). Moreover, when Nfkbia mRNA translation was inhibited by treatment with cycloheximide, the reduction in IκBα protein levels was more prominent in LICs than in non-LICs (Figure 5, D and E). These data indicate that the differences in IκBα levels are caused by the protein’s predominant degradation in LICs. Since both LICs and non-LICs are similarly exposed to high levels of TNF-α within leukemic BM cells, we considered that there would be differences in response to the stimulus and sequentially examined the downstream signals. We first hypothesized that there is a difference in TNF-α receptor expression levels between LICs and non-LICs that leads to greater TNF-α signal transmission in LICs. The expression patterns of TNF receptors I and II were, however, almost similar in LICs and non-LICs, although they varied between leukemia models (Supplemental Figure 8A). We next tested the phosphorylation capacity of IκB kinase (IKK) by examining the ratio of phosphorylated IκBα to total IκBα after treatment with the proteasome inhibitor MG132. Contrary to our expectation, a similar accumulation of the phosphorylated form of IκBα was seen in both LICs and non-LICs, implying that they had no significant difference in IKK activity (Supplemental Figure 8B). Another possibility is that the differences in IκBα protein levels are caused by predominant proteasome activity in LICs, because it is required for the degradation of phosphorylated IκBα. We measured 20S proteasome activity in LICs and non-LICs in each leukemia model by quantifying the fluorescence produced upon cleavage of the proteasome substrate SUC-LLVY-AMC and observed a 2- to 3-fold higher proteasome activity in LICs (Figure 5F). Furthermore, the expression of several genes encoding proteasome subunits was elevated in LICs compared with that in non-LICs (Figure 5G). Similarly, the published gene expression data on human AML samples revealed that CD34+CD38− cells had increased expression levels of proteasome subunit gene sets compared with those in CD34+ cells (Supplemental Figure 9 and ref. 30). These findings suggest that enhanced proteasome activity in LICs leads to more efficient degradation of IκBα in response to TNF-α, thus resulting in elevated NF-κB activity. We then tested the effect of bortezomib, a well-
known proteasome inhibitor, on LICs in vivo (Figure 5H). First, we treated mice with full-blown leukemia with a single injection of bortezomib and compared their BM surface-marker profiles with those of the vehicle-treated mice. Notably, bortezomib-treated mice showed a significant decrease in LIC-enriched populations in each type of leukemia (Figure 5, I and J). Finally, we treated mice with bortezomib after LIC transplantation and observed significant improvement in survival in those treated with bortezomib (Figure 5K). These results are very consistent with the selectively elevated proteasome activity we observed in LICs.

Enforced activation of the NF-kB pathway increases LIC frequency in leukemic BM. Given the supportive role of the NF-kB pathway in LIC proliferation as well as the differences in its activation status observed between LICs and non-LICs, we reasoned that the attenuation of NF-kB activity might be related to the transition from LICs to non-LICs. To test this hypothesis, we transduced MLL-ENL leukemia cells with a retrovirus encoding shRNA against IκBα and transplanted them into sublethally irradiated mice (Figure 6A). Because IκBα works as an inhibitor of NF-kB by holding it in the cytoplasm, its downregulation should function to...
enhance NF-κB activity, regardless of the basal proteasome activi-
ty. We first confirmed that MLL-ENL leukemia cells with shRNA-
mediated knockdown of IkBα (MLL-ENL-IkBαKD) showed
decreased IkBα protein levels in the cytoplasm and increased
nuclear p65 protein levels, which would indicate that NF-κB sig-
nal was enhanced by the reduction of its cytoplasmic inhibitor
(Figure 6B). In accordance with this finding, MLL-ENL-IkBαKD
cells had a significantly greater ability to secrete TNF-α than did
d control cells, reflecting an activated NF-κB/TNF-α signaling loop
(Figure 6C). We further investigated the phenotype of leukemic
mice with MLL-ENL-IkBαKD. Interestingly, the BM of these MLL-
ENL-IkBαKD mice showed a marked increase in immature Gr-1lo
c-Kit^b cell populations (Figure 6D). Consistent with this change,
we found that these leukemic cells had a greater CFC capacity
(Figure 6E). Additionally, in order to investigate the frequency of
LICs in BM mononuclear cells, we performed limiting dilution
analysis by secondary transplantation of leukemia cells. Although
the disease latency for leukemia development was not signifi-
cantly different among the leukemia cells, MLL-ENL-IkBαKD
leukemia cells had a marked abundance of LICs in the leukemic
BM mononuclear cells compared with the control shRNA cells
(Figure 6F and Supplemental Figure 10A). These data indicate
that enforced NF-κB activation expands the LIC fraction in MLL-
ENL leukemic BM cells. We also transduced normal BM cells with
shRNAs against IkBα and transplanted them into lethally irradi-
at mice to test whether NF-κB activation by itself can induce
leukemia or myeloproliferative-like disease. Over the 4-month
follow-up period, the mice exhibited no significant change in
peripheral blood values, indicating that NF-κB signal alone is not
sufficient for leukemogenesis (Supplemental Figure 10B).

Significant correlation between NF-κB and TNF-α is observed in
human AML LICs. Finally, we investigated NF-κB/TNF-α posi-
tive feedback signaling in human AML LICs. We analyzed CD34^-
CD38^ cells derived from 12 patients with previously untreated
or relapsed AML and the same cell population from 5 normal BM
specimens (Table 1) and evaluated their NF-κB signal intensity.
We also quantified the concentration of TNF-α in the culture
media conditioned by CD34^CD38^ cells from each patient in
order to measure the TNF-α secretory ability of these cells. As
expected, our data from both of these analyses showed a wide vari-
ation among patients, one that might reflect a heterogeneous dis-
tribution and frequency of the LIC fraction in human AML cells,
as was previously described (23). LICs in most of the patients did,
however, show increased p65 nuclear translocation and TNF-α
secretory potential compared with normal HSCs (Figure 7, A and
B, and Supplemental Figure 11). We plotted these two parame-
ters for each patient to compare between patients. Interestingly,
a significant positive correlation was demonstrated statistically
\( P = 0.02 \), as LICs with enhanced p65 nuclear translocation
showed a tendency toward abundant TNF-α secretion (Figure
7C). We also compared p65 intensity between LICs and non-
LICs in 2 patients (patients 1 and 3) and found that p65 nuclear
translocation was predominant in LICs, which is also consistent
with the data obtained in murine AML cells (Supplemental Fig-
ure 11). Moreover, we cultured LICs with or without neutralizing
antibodies against TNF-α and assessed p65 nuclear translocation
to determine the effect of autocrine TNF-α on NF-κB activity.
When incubated in the presence of TNF-α-neutralizing antibodi-
es, nuclear translocation of p65 was significantly suppressed in
LICs (Figure 7, D and E). These results support our hypothesis
that a positive feedback loop exists between NF-κB and TNF-α in
human AML LICs.

Discussion
In the present study, we provide evidence that LICs, but not nor-
mal HSPCs or non-LIC fractions within leukemic BM, exhibit
constitutive NF-κB pathway activity in different types of myeloid
leukemia models. Moreover, we identified the underlying mecha-
nism involved in the maintenance of this pathway activity, which
had yet to be elucidated. We found that autocrine TNF-α secre-
tion, with the support of enhanced proteasome activity, contrib-
uted to a constitutive activation of the NF-κB pathway in LICs.
Although we observed different sensitivities to the inhibition of
these signaling cascades according to the type of leukemia, these
cascades play an important role in LIC proliferation, especially
considering that the complete ablation of Tnf or Rela distinctly
suppressed leukemia progression in vivo. These findings, which
we validated in human AML LICs, could translate into improved
AML treatment strategies.

The strong connection between inflammation and cancer has
been increasingly discussed, and the NF-κB pathway is now rec-
ognized as a major regulator bridging the two pathological con-
ditions in different types of malignancies. In most of these malig-
nancies, aberrant activation of the NF-κB pathway derives from
inflammatory microenvironments that are mainly created by
proinflammatory immune cells such as tumor-infiltrating macro-
phages, neutrophils, and lymphocytes (34, 35). In this study,
however, LICs retained their p65 nuclear translocation even after
steroid-free culture, suggesting that the constitutive NF-κB activ-
ity of LICs is maintained in an autonomous fashion. Through
our investigation of gene expression profiles in LICs and normal
HSCs, we found that LICs had distinctly elevated TNF-α expres-
sion levels that contributed to the maintenance of NF-κB activa-
tion in LICs. Conversely, the introduction of IkB-AS markedly
suppressed TNF-α expression levels, indicating that NF-κB activ-
ity and TNF-α secretion create a positive feedback loop in LICs.
Moreover, our hypothesis is strongly supported by our findings
that a positive correlation exists between NF-κB and TNF-α secre-
tory activities in human AML CD34^CD38^ cells and that inhibi-
tion of autocrine TNF-α signaling attenuates p65 nuclear translo-
cation. The role of TNF-α in the process of tumor promotion
has recently been demonstrated in various types of solid tumors
(36–39). It has also been reported that TNF-α is required for clonal
evolution of myeloid malignancies (40). On the other hand, there
has been controversy over the effect of TNF-α on leukemia cells
when it was exogenously administered (41, 42). However, these
previous studies did not address the critical question of whether
endogenously secreted TNF-α is required for the maintenance of
established leukemia cells, which is a crucially important aspect
when considering therapeutic applications. We clearly reveal that
the autonomously secreted TNF-α had beneficial effects on LIC
proliferation through NF-κB activation, while the contribution of
paracrine TNF-α secretion from BM microenvironments was min-
imal. Another important aspect of cytokine secretion by LICs that
was not investigated in the present study is whether this secretion
can exert some influence on BM stromal cells. Since the impor-
tance of bidirectional crosstalk between leukemia and niche cells
through a variety of cytokines has increasingly been recognized
(43), TNF-α secreted from LICs might also modulate the function of
BM stromal cells, which could also have an impact on leukemia
progression. Unveiling the role of TNF-α as a paracrine mediator would further extend the therapeutic options for AML.

Few studies have compared the NF-κB activity of different fractions within leukemia cells, and the mechanism underlying the difference in this activity has not been analyzed (44). We focused on proteasome activity as the essential machinery supporting NF-κB activity in LICs. Although high proteasome activity has been reported in various types of cancers (45, 46), its actual role in the malignant phenotype remained to be elucidated. In this study, we found that proteasome activity was especially high in LICs, which contributed to selective NF-κB activity in LICs via the efficient degradation of IkBα. Conversely, the inefficient NF-κB nuclear translocation we observed in non-LICs, despite TNF-α-enriched leukemic BM cells, could be explained by the low proteasome activity in these cells. Therefore, we postulate that both an activating stimulus such as TNF-α and high proteasome activity are required for efficient NF-κB signaling (Figure 7F). Both of these conditions are present exclusively in LICs, which acquire selective NF-κB activation. We also found that the expression levels of proteasome subunit genes were elevated in LICs compared with those in non-LICs, genes that could be involved in regulating proteasome function. Because we observed similar expression patterns in LICs and non-LICs in human AML cells, an elevated expression level of proteasome subunit genes might be one of the common characteristics of the LIC phenotype. Further studies will be needed to elucidate the regulatory mechanism of the proteasome gene families.

Our findings provide several advantages when considering their application to the clinical care setting. First, an activated NF-κB/TNF-α feedback loop was seen in AML LICs that had different genetic abnormalities. Although the therapeutic strategy of targeting aberrant molecules based on genetic abnormalities such as FLT3-TTD is promising, its application is limited to a particular group of patients. In contrast, inhibition of the NF-κB signal in addition to standard chemotherapy would show beneficial effects in most AML patients. Second, because there was a strong positive correlation between the NF-κB signal and TNF-α secretion, therapeutic efficacy could easily be inferred from the abundance of TNF-α instead of from evaluation of the activation status of NF-κB. Third, the NF-κB/TNF-α signal and enhanced proteasome activity are selectively seen in LICs, but not in normal HSCs. A recent study has shown that complete ablation of p65 in hematopoietic cells attenuates the long-term capacity for hematopoietic reconstitution (47). However, our data from the experiments in which we introduced IkB-SR into normal BM cells show that partial repression of NF-κB activity exerted minimal influence on normal hematopoiesis, while it markedly inhibited leukemia progression. These results indicate that there is a therapeutic window during which LICs can selectively be killed by NF-κB inhibition without seriously affecting normal hematopoiesis. Alternatively, there is some evidence that TNF-α has suppressive effects on normal HSCs (48, 49). The opposing role of TNF-α in LICs and HSCs is additionally beneficial, since anti–TNF-α therapy contributes to the recovery of normal hematopoiesis and attenuates LIC proliferation. Now that the TNF-α antagonist etanercept is widely used in inflammatory diseases such as rheumatoid arthritis, this drug might be a promising candidate for treating patients with AML.

In summary, the present study shows that blocking the NF-κB pathway offers a promising therapeutic approach for targeting LICs in various types of myeloid leukemia, without disturbing normal hematopoiesis. We further determined that autocrine TNF-α signaling and enhanced proteasome activity are crucial for maintaining constitutive NF-κB activity in LICs, findings that may also provide a new therapeutic opportunity.

Methods

Animals. C57BL/6 mice and BALB/c mice were purchased from Japan SLC, Inc. Tnf-knockout mice on a BALB/c background were established as described previously (50). Rela-floxed mice on a C57BL/6 background were provided by H. Algul and R.M. Schmid (32). BALB/c mice were used as the controls in the experiments using Tnf-knockout mice, and C57BL/6 mice were used in the other experiments.

Retrovirus production and BM transplantation assays. To obtain retrovirus supernatants, platinum-E (Plat-E) packaging cells were transiently transfected with retroviral vectors and supernatants, platinum-E (Plat-E) packaging cells were transiently transfected with retroviral vectors and retained for 48 hours after transfection and used immediately for infection. To establish each myeloid leukemia mouse model, we used pMSCV-neo-MLL-ENL; pMSCV-MLL-ENL-internal ribosome entry site-EGFP (IRES-EGFP); pGCDNsam-MLL-ENL-IRES-Kusabira-Orange; pGCDNsam-MOZ-TIF2-IRES-EGFP; pGCDNsam-MOZ-TIF2-IRES-Kusabira-Orange; pGCDNsam-BCR-ABL-IRES-EGFP; pGCDNsam-BCR-ABL-IRES-Kusabira-Orange; and pMSCV-neo-NUP98-HOXA9. GMPs isolated from the BM of 8- to 10-week-old mice were transduced with the respective vectors and injected into sublethally irradiated (7.5 Gy) recipient mice. For experiments involving the generation of leukemia cells with IkB-SR, MLL-ENL leukemia cells were transduced with pBABE-GFP or pBABE-GFP-IkB-SR. MOZ-TIF2, and BCR-ABL-NUP98-HOXA9 leukemia cells were transduced with pGCDNsam-Kusabira-Orange or pGCDNsam-IkB-SR-IRES-Kusabira-Orange. For experiments involving the deletion of p65 in Rela-floxed mice, leukemia cells were established using Kusabira-Orange-containing retroviral vectors. The developed leukemia cells were transduced with pGCDNsam-EGFP or pGCDNsam-iCre-EGFP and transplanted into sublethally irradiated mice.
measurements were performed using FluoView software. The background-subtracted intensity ratio of nucleus/cytoplasm was calculated in more than 50 cells in each specimen, and the average intensity with SD is presented.

Flow cytometry. Isolation of each fraction from normal or leukemic BM cells was performed using a FACSAria II (BD) cell sorter. For isolation of GMPs and KSLs, biotinylated antibodies against Gr-1 (RB6-8C5), CD11b (M1/70), B220 (RA-3-6B2), CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), and TER119 were used for lineage staining. A PerCP-Cy5.5–labeled streptavidin antibody was used for secondary staining, together with APC–anti–c-Kit (2B8), PE-Cy7–anti–Sca-1 (E13-161.7), FITC–anti-CD34 (RAM34), and PE–anti-CD16/32b antibodies (clone 93). The following antibodies were used for isolation of L-GMPs from GFP-containing leukemia cells: APC-Cy7–anti–streptavidin, PE-Cy5–anti–c-Kit (2B8), PE-Cy7–anti–Sca-1 (E13-161.7), Alexa Fluor 647–anti–CD34 (RAM34), and PE–anti–CD16/32b (clone 93). APC-antistreptavidin and PE-Cy7–anti–Sca-1 antibodies (E13-161.7) were used for sorting LICs and non-LICs in the BCR-ABL plus NUP98-HOXA9 leukemia model. See Supplemental Figures 1 and 2 for detailed FACS plots. For analysis of TNF receptor expression in leukemia cells, biotinylated antibodies against TNF receptor I or II (55R-170) and an APC-Cy7–anti–streptavidin antibody were used. Analysis was performed using FlowJo software (Tree Star Inc.).
Table 1
Clinical characteristics of the 12 patients with AML and the 5 patients with normal BM findings

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Sex</th>
<th>BM findings</th>
<th>Disease status</th>
<th>Type</th>
<th>Cytogenetics</th>
<th>Blast (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>M</td>
<td>AML</td>
<td>Untreated</td>
<td>M2</td>
<td>Normal</td>
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<tr>
<td>2</td>
<td>62</td>
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<td>AML</td>
<td>Relapse</td>
<td>M1</td>
<td>47, XY, del(9)(q13q22), +10</td>
<td>96</td>
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<td>69</td>
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<td>AML</td>
<td>Untreated</td>
<td>M4</td>
<td>Normal</td>
<td>90</td>
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<td>M</td>
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<td>Untreated</td>
<td>M3</td>
<td>46, XY, t(15;17)</td>
<td>63</td>
</tr>
<tr>
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<td>M4</td>
<td>46, XY, inv(16)</td>
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<td>6</td>
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<td>F</td>
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<td>AML-MRC</td>
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<td>46, XY, t(11;17)</td>
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<td>M1</td>
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<td>46, XY, t(1;3)</td>
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Real-time quantitative PCR. Real-time quantitative PCR was carried out on the LightCycler480 system (Roche) using SYBR green reagents according to the manufacturer’s instructions. The results were normalized to Gapdh levels. Relative expression levels were calculated using the 2-ΔΔCt method (51). The following primers were used for real-time PCR experiments: Gapdh forward, TGGCCTCAAGAGATGAA; and reverse, GGTCGGAGGAAATTGTG; Nf2 forward, CCAGAAGACCTGGAAATTGTG, and reverse, AAATGCACATTCCCTCCCTACA; Tnf forward, TCTTCTCATCTGCTGGTGG, and reverse, GGTCGCGCCCATGACTGA; H11A forward, TAAACTCAACGACAGGACTTGA; reverse, GGGTGCCTGCTGCAGTGTAT, and reverse, GGTAGTCACCTTTGGTGTCA; Lif forward, CTCCAGAGGCTTACACAA, and reverse, AGGTGAGATTCTTCCCTTC; Hexα forward, GGTAAGCTCTGCAATGTGAT, and reverse, GTCTGACCAAGGACCATAAT; Pim3 forward, CGAGTACAGGAGGGTTGTG, and reverse, TGGATGCAGATGCTGTAG; Pimmd4 forward, GTATAGCCGGAAGGACT, and reverse, TTGAGTCGACGACCTCAG; Pim3 forward, TTTAGATGAGGGAATCACA, and reverse, GGTCTGATAATTGAAAATGTTTC.

siRNA interference. Specific shRNAs targeting murine Iκbα mRNA were designed and cloned into pSIREN-RetroQ-ZaGreen vectors. Control shRNA is a nonfunctional construct provided by Clontech. The target sequences, from 5’ to 3’, were: CCGAGACTTTGCGAGAAAT (shIkBa number 1), and AGCTGACCTGGAATATCCT (shIkBa number 2).

Immunoblotting. Membranes were probed with the following antibodies: anti-IκBα (Cell Signaling Technology), anti–phospho-IκBα (Ser32) (Cell Signaling Technology), anti–phospho-p65 (Santa Cruz Biotechnology Inc.), anti–phospho-p65 (Ser536) (Cell Signaling Technology), anti–β-actin (Cell Signaling Technology), and anti–histone H3 (Cell Signaling Technology). Protein levels were quantified with ImageJ software (NIH). To obtain nuclear and cytoplasmic extracts, an Active Motif Nuclear Extract Kit was used according to the manufacturer’s instructions. Cycloheximide treatment assay was performed as described previously, with modification (52). Cells were pretreated with MG132 (20 μM) for 1 hour to initially inhibit the proteasomal degradation of IkBα. Cells were washed twice with medium, then cultured with or without 10 μg/ml of cycloheximide for an additional hour and harvested. AML or normal CD34+CD38− cells were cultured for 48 hours in RPMI medium containing 10% FBS and 100 ng/ml IL-3, and thrombopoietin. The concentration of TNF-α in the harvested supernatants was measured with a murine TNF-α Quantikine ELISA kit (R&D Systems).

20S proteasome activity. A 20S proteasome activity assay kit (Cayman Chemical) was used to analyze proteasome activity. A total of 5×10⁴ freshly isolated normal GMPs, LICs, and non-LICs in each model were assayed according to the manufacturer’s protocol. As a control, the proteasome activity of each cell was also assayed after the specific proteasome inhibitor epigallocatechin gallate was added. Fluorescence was measured with a Wallac ARVO V (PerkinElmer), and the proteasome activity of each cell type was calculated by subtracting the respective control value.

Bortezomib treatment studies. For in vivo treatment experiments, LICs of each leukemia model were injected into sublethally irradiated mice: 1×10⁶ cells in the MLL-ENL or BCR-ABL/NUP98-HOXA9 models, and 1×10⁴ cells in the MOZ-TIF2 model. Bortezomib was administrated i.p. at doses of 1.0 mg/kg twice weekly for 3 weeks. Treatment was started 1 week after transplantation in the MLL-ENL or BCR-ABL/NUP98-HOXA9 models, and 2 weeks after transplantation in the MOZ-TIF2 model. For experiments analyzing changes in LIC populations, bortezomib was administrated i.p. at doses of 1.0 mg/kg into fully developed leukemic mice. GFP+ BM cells were collected 24 hours after injection, and surface marker profiles were analyzed.

Analysis of microarray data. We analyzed publicly available gene expression microarray data on murine and human samples from the Gene Expression Omnibus (GEO) database (GEO GSE24797, GSE20377, and GSE4006). A set of CEL files were downloaded from GEO and normalized using the JustRMA function from the Affy package 1.22.1 in Bioconductor. To compare expression profiles of the NF-κB target genes, normalized data were tested for GSEA using previously described NF-κB target gene sets (29), and a nominal P-value was calculated. For screening of genes with elevated expression levels in LICs compared with those in normal HSPCs, the expression values of individual genes were compared between groups. Genes significantly elevated in LICs from all three leukemia models as determined by an unpaired Student’s t test (P < 0.05)
Figure 7
NF-κB/TNF-α positive feedback loop is activated in human AML LICs. (A) Quantification of p65 nuclear translocation assessed by the mean nucleus/cytoplasm intensity ratio by immunofluorescence staining. The CD34+/CD38– fractions isolated from AML or normal BM were analyzed. More than 50 cells were scored in each specimen, and the average intensity ratio with SD is shown. (B) TNF-α concentration of culture media conditioned by human AML LICs and normal HSCs measured by ELISA. ND, not detected. (C) Correlation between p65 nuclear translocation intensity ratio and TNF-α secretory ability of human AML LICs. (D) Immunofluorescence assessment of p65 nuclear translocation in LICs purified from 4 patients after serum-free culture with neutralizing antibody against TNF-α or isotype control. Scale bars: 10 μm. (E) Quantification of p65 nuclear translocation of LICs with or without neutralizing antibody against TNF-α assessed by the mean nucleus/cytoplasm intensity ratio. (F) Proposed model showing the role of NF-κB signaling in LICs. Positive feedback loop involving NF-κB/TNF-α promotes the maintenance and proliferation of LICs. The signaling is supported by active proteasome machinery, which declines with LIC differentiation.
were selected, among which genes also elevated in human AML LICs (Stu-
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