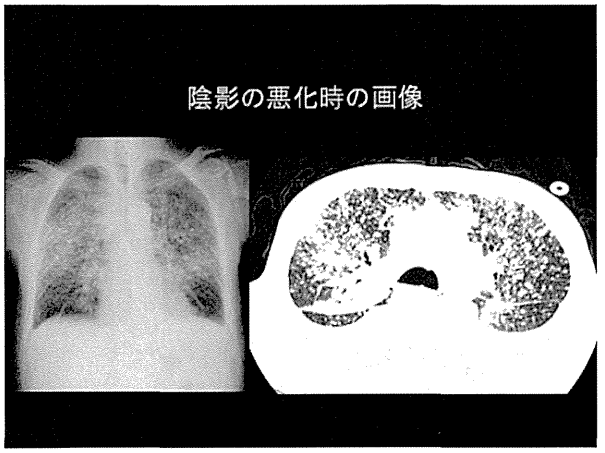
【画像所見】



【入院後経過】

胸腔鏡下の中葉部分切除術を施行して、瘢痕様線維組織内に泡沫組織球の増生，活動性線維芽細胞増生と泡沫組織球の混在を認める。Erdheim Chester 病，Histiocytosis X, Rosai-Dorfman 病が鑑別になった。免疫染色にて、CD1a—， langerin—，S-100—，第 VIIIa+, CD163+にてエルドハイムチェスターの診断． PSL + CyA 投与で、陰影はやや改善する。

F .研究発表

なし

G．知的財産権の出願・登録状況

1. 特許取得

「該当なし」

2. 実用新案登録

「該当なし」

3.その他

「該当なし」

厚生労働省科学研究費補助金（難治性疾患政策研究事業）
「Erdheim-Chester 病に関する調査研究」分担研究報告書

希少疾患領域の研究デザインに関する研究

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研究要旨
Erdheim-Chester disease (ECD)は、非ランゲルハンス細胞性組織球症の一型で、全身に浸潤した組織球により骨痛、腎不全、心不全、肺線維症、尿崩症、眼球突出など多彩な症状を呈する疾患で、6割の患者が32ヶ月以内に死亡するとの予後不良な疾患である。世界的に見ても数百例程度の希少疾患であり、標準的治療法も改善されていないなど不明な点が多く存在する。本研究に関し、平成26年度は科横断的にECD症例情報を集積し、有病率、臨床症状、病変部位別の頻度等の基礎的なデータをまとめ、本邦におけるECD診療の実態を把握した。さらに、得られたデータより発症関連因子や予後関連因子などの解明を通じて重症度分類の確立、治療指針の作成を行い、ECD患者の診断及び治療の一助とすることが最終的な目標である。当研究室は、このような希少疾患による疾患の発生動向を確認する為の研究デザインの組み方とデータ管理の方法論について担当した。

A．研究目的

Erdheim-Chester disease (ECD)は、非ランゲルハンス細胞性組織球症の一型で、全身に浸潤した組織球により骨痛、腎不全、心不全、肺線維症、尿崩症、眼球突出など多彩な症状を呈する疾患で、6割の患者が32ヶ月以内に死亡するとの予後不良な疾患である。世界的に見ても数百例程度の希少疾患であり、標準的治療法も改善されていないなど不明な点が多く存在する。本研究に関し、平成26年度は科横断的にECD症例情報を集積し、有病率、臨床症状、病変部位別の頻度等の基礎的なデータをまとめ、本邦における ECD 診療の実態を把握した。さらに、得られたデータより発症関連因子や予後関連因子などの解明を通じて重症度分類の確立、治療指針の作成を行い、ECD患者の診断及び治療の一助とすることが最終的な目標である。当研究室は、このような希少疾患による疾患の発生動向を確認する為の研究デザインの組み方とデータ管理の方法論について担当した。

B．研究方法

希少疾患領域の特殊性を考慮した、前向きコホート研究などの疫学データの蓄積や臨床研究を実施する上での方法論を検討し、必要なデータ管理の標準化、効率化に関する考察を加える。

（倫理面への配慮）

本研究は、遺伝子変異を解析する研究であり、人権擁護上対象者に対する配慮が必要である。このことより、当研究は、ヒトゲノム・遺伝子解析研究に関する倫理指針、疫学研究に関する倫理指針、臨床研究に関する倫理指針を遵守するとともに、事前に施設の倫理審査委員会の承認を得て行う。尚、本研究で用いる臨床検体は、患者の臨床的診断のために採取された検体の残検体を用いる為、健康上の危険性は殆どないが、本研究の為の解析に際しては、書面にてインフォームド・コンセントを得て行う。また、臨床情報及び検体の取扱いについては、参加施設の規定に従い、匿名化を図り行う。以上の手続きをとることにより、研究の

安全性及び被験者の倫理瀬的妥当性が確保される。

C．研究結果

当研究室では小児希少疾患のデータ管理の実務・研究の経験をもとに、希少疾患に対する疫学研究と臨床試験を融合させた研究体制を構築し、その上で臨床試験不参加例の詳細な分析や対象患者の長期的観察を実現するための枠組みを電子的データ収集システム(EDC)開発と共に構築してきた。これは希少疾患領域における研究開発を進める上で今後の研究推進に役立つ、不参加例の分析や臨床試験を実施する為の知見を得ることなどの強みがあると考えている。その他の研究開発の方向性としては、国際共同試験への参画による症例数確保や、国内での小規模臨床試験の実施（特に医薬品の承認など開発戦略を組んだ方式を模索する場合）などがあることを確認した。

一方、臨床研究デザインに応じて、データ管理の方法論は異なり、これにかかるコストの振れ幅は大きくも小さくもなる。特に希少疾患など市場が小さい疾患領域においては、資金確保が困難であることから、臨床研究にかかるコストの最適化は限りある資源の最適配分を考える上でも深刻な問題である。近年、O'Leary E. らは、収集項目のうち論文化に使用された項目は僅か18%であることを示した。取得データの最適化を目指し、研究のエンドポイントについて、解析に使用するデータと、試験の品質を担保するために使用するデータを分けて詳細に分析した。具体的には、CDISC (Clinical Data Interchange Standards Consortium)により標準化された SDTM (Study Data Tabulation Model)という、米国食品医薬品局による再解析データ用に最適化されている変数へのマッピングの可能性について、小児血液疾患臨床試験領域の 3 臨床試験で取得したデータ、各試験について論文化された 8 報告の中で使用されたデータを用いて検討した。試験の特性を表 1 に示す。

表 1. 臨床研究特性

試験	対象疾患	Phase	Primary Endpoint	対象年齢	試験治療期間	予定症例数	登録期間	追跡期間	論文数
A	急性リンパ性白血病	後期II相	18m EFS	<1y	6M	70	5年	3年	1
B	急性骨髄性白血病	後期II相	3y EFS	<18y	5-6M	254	4年	3年	5
C	リンパ腫	後期II相	2y EFS	<18y	4-6M	308	6年	2年	2

解析用データフィールドを CDISC SDTMIGver3.2 の Req 変数名にマッピングできる数をカウントし、その割合を算出した（各ドメインには入力必須で null を許容しない変数がある）ところ、表 2 に示すように全フィールドに対する解析用フィールド、中央モニタリング用フィールドは各々24%, 76%であった。

表 2. CDISC SDTMIGv3.2 へのマッピング状況

試験名	全フィールド数	中央モニタリング	(%)	解析	(%)
A	434	325	75%	109	25%
B	550	453	82%	97	18%
C	340	225	66%	115	34%
平均	441	334	76%	10	24%

項目別検討より、解析用フィールドは、毒性、フォローアップの順に多く、また解析用フィールドは、すべて CDISC SDTM の変数にマッピングが可能であることがわかった(19/38 ドメイン)。解析用フィールドは、全ドメインの入力必須変数(Req)を、全て網羅していた。

以上より、まずエンドポイントに直接関係しない中央モニタリングフィールドの最適化が図れれば、大幅なコスト削減が図れる可能性が示せた。また、エンドポイントに直接関係する解析用データは、臨床データの国際標準である CDISC に適合しており、国際共同研究等のメタ解析に活用できる可能性が示された。3 試験共に解析用フィールドが Req 変数を網羅していたことは、SDTMIGver.3.2 の eCRF プレマップ機能を搭載した EDC 活用の可能性に繋がると考えた。Risk-based approach を念頭においた、中央・施設モニタリング用のデータ取得量の最適化により、品質を確保しながら効率化が図れる可能性が示された。

D．考察

ECD を始めとした希少疾患を対象とした臨床試験を計画する上で、限りある資源の適正配分を考

慮した場合、臨床研究デザイン上の工夫とデータ管理（中央モニタリング）・施設モニタリングの工夫が必要となる。特にデータ管理の方法論の検討より、質を維持したままデータ管理を効率化できる可能性を示した。

E．結論

ECD など希少疾患を対象とした臨床試験を計画する上で、適切な臨床研究デザインを企てると共に、データ管理方法の最適化を計画することにより、質を維持したまま妥当なコストでデータ管理できる可能性が示された。今後も引き続き希少疾患領域における研究の基盤整備に努める。

F．研究発表

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日本臨床試験学会　第 6 回学術集会総会
2015.2.20 (東京)

G．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
   該当なし
2. 実用新案登録
   該当なし
3. その他
   該当なし

III. 研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
村上有香子 片山一朗	組織球症. 幼少児によくみられる皮膚疾患アトラス－鑑別と治療のポイント	片山一朗 横関博雄	幼小児によくみられる皮膚疾患アトラス-鑑別と治療のポイント	医薬ジャーナル社	日本	2015	158-159

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kagoya Y, Yoshimi A, Kataoka K, Nakagawa M, Kumano K, Arai S, Kobayashi H, Saito T, Iwakura Y, Kurokawa M.	NF-κB/TNF-α positive feedback loop with active proteasome machinery supports myeloid leukemia initiating cell capacity.	J Clin Invest.	124	528-542	2014
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Tsurusawa M, Gosho M, Mori T, Mitsui T, Sunami S, Kobayashi R, Fukano,R, Tanaka F, Fujita N, Inada H, Koh K, Takimoto T, Saito A, Fujimoto,J, Nakazawa A, Horibe K; for the lymphoma committee of the Japanese Pediatric Leukemia/lymphoma Study Group.	Statistical analysis of relation between plasma methotrexate concentration and toxicity in high-dose methotrexate therapy of childhood nonHodgkin lymphoma.	Pediatr Blood Cancer.			2014
Koh K, Tomizawa D, Saito AM, Watanabe T, Miyamura T, Hirayama M, Takahashi Y, Ogawa A, Kato K, Sugita K, Sato T, Deguchi T, Hayashi Y, Takita J, Takeshita Y, Tsurusawa M, Horibe K, Mizutani S, Ishii E.	Early use of allogeneic hematopoietic stem cell transplantation for infants with MLL gene-rearrangement-positive acute lymphoblastic leukemia.	Leukemia.	29(2)	290-6	2015
Manabe A, Kawasaki H, Shimada H, Kato I, Kodama Y, Sato A, Matsumoto,K, Kato K, Yabe H, Kudo K, Kato M, Saito T, Saito AM, Tsurusawa M, Horibe K.	Imatinib use immediately before stem cell transplantation in children with Philadelphia chromosome-positive acute lymphoblastic leukemia:Results from Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) Study Ph+ ALL04.	Cancer Med.			2015

IV. 研究成果の刊行物・別刷
(主なもの)

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

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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 proteasome machinery, which promoted the degradation of IκBα 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.

Introduction

Acute myeloid leukemia (AML) is a highly aggressive hematologic malignancy characterized by a relentless proliferation of immature myeloid blasts. Recent studies have demonstrated that the apparently uniform leukemia cell population is organized as a hierarchy that originates from leukemia-initiating cells (LICs) (1, 2). Although intensive chemotherapy is initially effective in most cases of AML, the surviving LIC clones repopulate the disease, leading to subsequent relapse and an ultimately dismal prognosis (3). Another problem is that AML is a heterogeneous disease with different cytogenetic and molecular abnormalities. This heterogeneity has increasingly been unveiled by recent work involving the screening of recurrent mutations seen in AML cells using high-throughput sequencing technology, which is useful for constructing individualized therapeutics (4, 5). At the same time, however, these findings indicate that it is difficult to develop a treatment strategy in addition to standard chemotherapy that is widely applicable to AML. Therefore, to establish effective treatments, it is important to identify the universally essential mechanisms involved in the LIC phenotype, irrespective of the cells' diverse genetic abnormalities.

NF-κB is a transcription factor initially discovered in B cells (6). Although well known for its role in controlling various aspects of immune responses, the NF-κB pathway is now also recognized as an important regulator of cell survival, proliferation, and differentiation (7–9). Its constitutive activation has been reported in a variety of malignancies and mostly plays a cancer-promoting role (10–12). There is some evidence that this pathway activity is also seen in the AML $CD34^+CD38^-$ fraction, which is considered to be enriched for LICs (13, 14). Given that NF-κB activity is not restricted to specific AML subtypes or genetic abnormalities, it is possible that the signaling is universally essential for myeloid leukemia progression, and a variety of agents have been reported to induce apoptosis in cultured leukemia cells via NF-κB pathway inhibition (15–19). The effect of specific inhibition of the NF-κB pathway on LICs in vivo, however, has not been sufficiently studied. Furthermore, the mechanism of this pathway's activation remains to be elucidated. Although several gene mutations found in hematologic malignancies have been reported to be associated with enhanced NF-κB signaling (20–22), these findings do not fully explain why the activation of NF-κB is observed in a number of different types of leukemia. It is more intriguing, as well as reasonable, to consider that NF-κB activation arises from the signaling pathways that are commonly involved in LICs. Another limitation of the previous studies is that LIC-enriched populations in AML are highly heterogeneous among patients and are not necessarily confined to the $CD34^+CD38^-$ fraction, as they are in normal HSCs. Therefore, it is problematic to strictly define LICs by their surface-marker antigens (23, 24).

To overcome these challenges, we used variable myeloid leukemia mouse models, in which LIC-enriched fractions were well characterized using a surface marker phenotype and revealed that NF-κB signaling is constitutively activated in LICs, but not in normal cells or non-LIC fractions within leukemic BM cells. We also elucidate the mechanism of NF-κB activation in LICs in each model and demonstrate that the inhibition of NF-κB or its upstream machinery in LICs markedly suppresses leukemia progression in vivo.

Results

The NF-κB pathway is activated in LICs of different types of myeloid leukemia models. To extensively investigate NF-κB activity in LICs of

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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 cell populations that would barely contain LICs, we also sorted lineage$^-$ c-Kit$^-$ cells in MLL-ENL and MOZ-TIF2 leukemic mice and lineage$^+$ cells in a BCR-ABL/NUP98-HOXA9 model. There were striking differences in clonogenic potential (Supplemental Figure 3) and LIC frequencies, as determined by in vivo limiting dilution assays in the two populations of each model (Figure 1A and Supplemental Table 1). Therefore, we confirmed that LIC and non-LIC fractions can be clearly isolated through the surface antigen profiles of the three leukemia models. Next, we visualized the subcellular distribution of the major NF-κB subunit p65 in LICs, non-LICs, and normal cells by immunofluorescence staining and confocal microscopy. As shown in Figure 1B, prominent nuclear translocation of p65 was observed in the LICs of each model, while it was retained mostly in the cytoplasm in normal lineage$^-$ c-Kit$^+$ Sca-1$^+$ cells (KSLs), which are enriched for HSCs and GMPs. Interestingly, non-LICs also had relatively reduced p65 nuclear translocation signal compared with that in LICs in all three leukemia models. We quantified the nucleus/cytoplasm ratio of p65 staining intensity in these images, which also showed that the LICs in each model had significant nuclear localization compared with that observed in non-LICs, normal KSLs, and GMPs (Figure 1C).

To further test NF-κB transcription activity in LICs, we investigated the expression profiles of a subset of genes regulated by the NF-κB pathway. We first used two sets of published gene expression microarray data, which compared the expression profiles of MOZ-TIF2 L-GMPs (26), MLL-AF9 L-GMPs, and HOXA9-MEIS1 L-GMPs (28) with those of normal hematopoietic stem or progenitor cells (HSPCs). The expression profiles of previously identified NF-κB target genes were assessed by gene set enrichment analysis (GSEA) (Supplemental Table 2 and ref. 29), which showed that L-GMPs had increased expression levels of NF-κB target genes compared with those in normal HSPCs in both sets of gene expression microarray data (Figure 2A). We also compared the expression profiles of the same gene set in CD34$^+$CD38$^-$ human AML cells with those of the equivalent cell population in normal BM cells, which corresponded to the HSC fraction, and observed a similar tendency (Figure 2B and ref. 30). Then, we validated these results using quantitative real-time PCR by comparing the expression levels of several NF-κB target genes in LICs and non-LICs from our three mouse models with those in normal GMPs and found increased expression levels of most of the genes in different types of LICs, but no significant elevation of these levels in non-LICs (Figure 2C and Supplemental Figure 4). Furthermore, the level of p65 phosphorylation, which is important for enhancing its transcription activity, was significantly increased in LICs compared with the level observed in normal GMPs (Figure 2D). Consistent with these findings, LICs showed a more prominent increase in apoptosis than did normal cells or non-LICs when treated with sc-514, a selective inhibitor of IκB kinase β (IKKβ) (Figure 2, E and F, and ref. 31). Although LICs from BCR-ABL/NUP98-HOXA9–induced leukemia were rather resistant to sc-514 compared with cells from MLL-ENL– and MOZ-TIF2–induced leukemia, they still showed higher sensitivity than non-LICs. Collectively, these data fully support the hypothesis that the NF-κB pathway is constitutively activated in the LICs of different types of myeloid leukemia.

LICs maintain their constitutive NF-κB activity via autocrine TNF-α signaling. In the next step, we addressed the question of how LICs maintain constitutive NF-κB activity in different types of leukemia models. In order to investigate genes prevalently dysregulated in LICs, we analyzed the previously published microarray-based gene expression profiles comparing murine and human LICs with normal HSPCs (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 that 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 subcellular distribution. 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 fashion.

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*-deficient 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*-deficient leukemia cells failed to effectively establish AML in

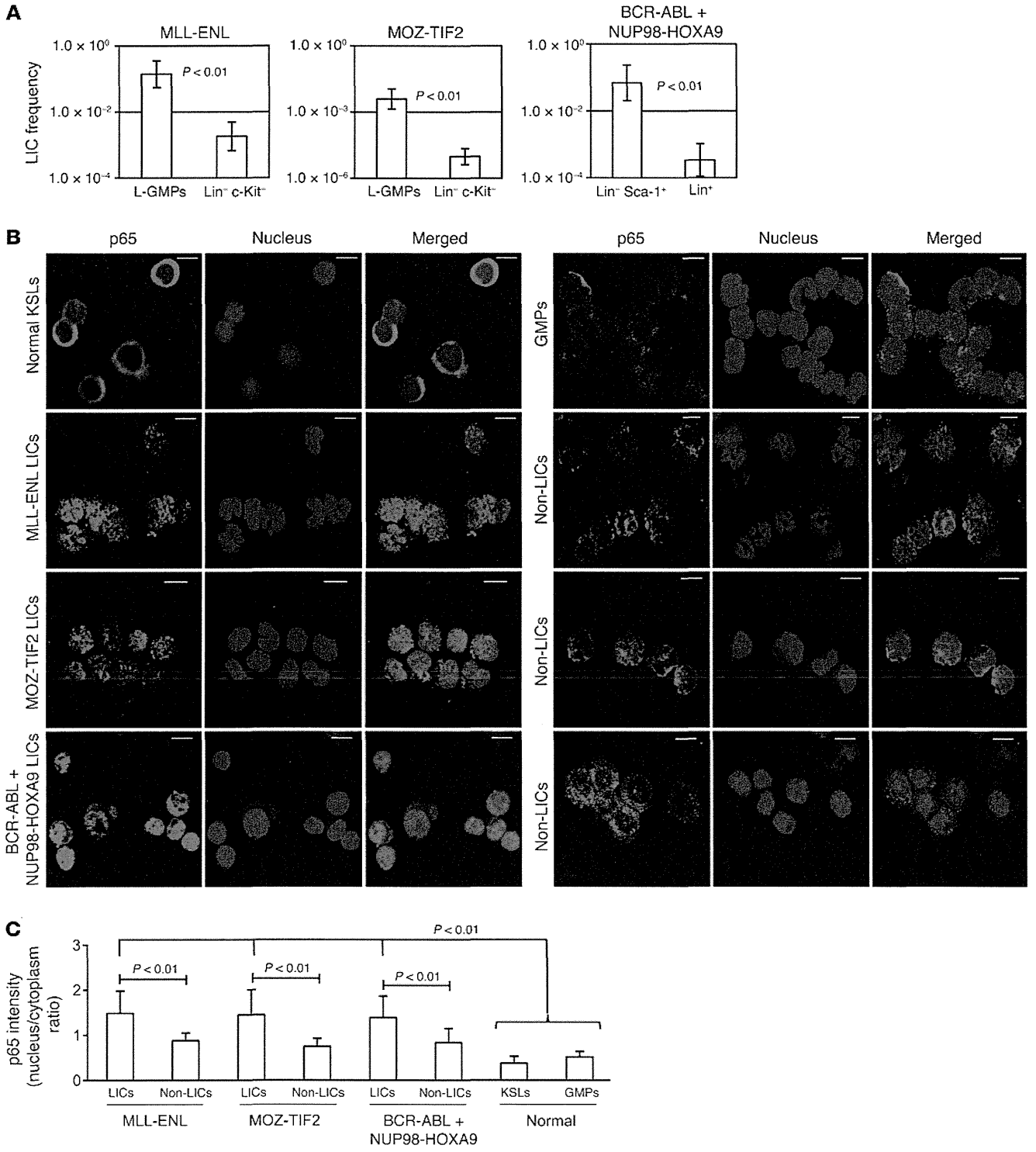

Figure 1

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.

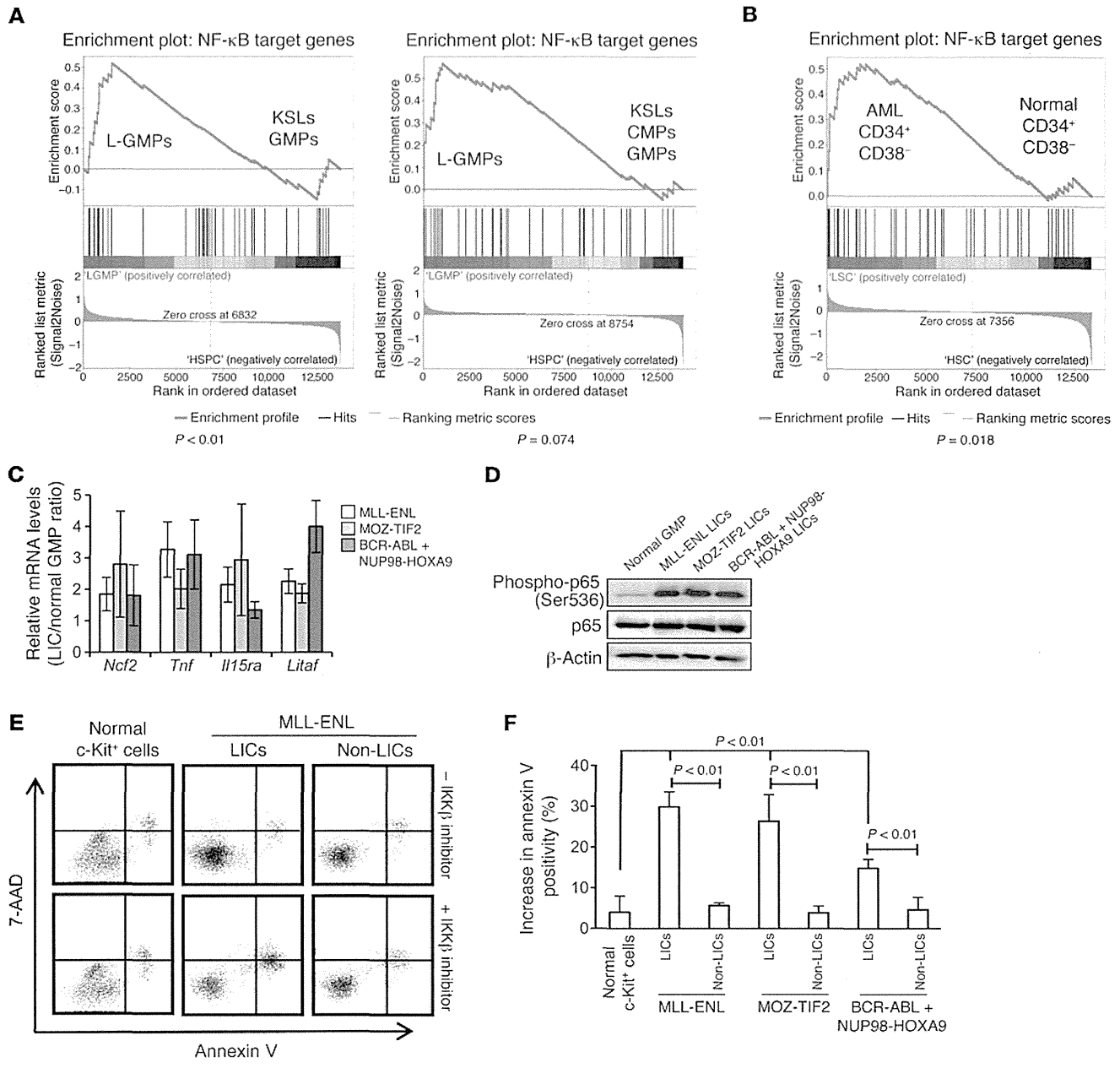

Figure 2

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 $c\text{-}Kit^+$ cells, L-GMPs, and $Lin^-c\text{-}Kit^-$ 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 $c\text{-}Kit^+$ cells treated with 10 μM IKK inhibitor (sc-514) ($n = 4$ each). Error bars indicate SD.

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.

The impact of specific NF-κB inhibition on leukemia progression. To investigate the influence of specific NF-κB pathway inhibition on leukemia progression in vivo, we transduced MLL-ENL leukemia cells with a retroviral vector expressing a dominant-negative form of IκBα (super repressor, referred to herein as IκB-SR) or

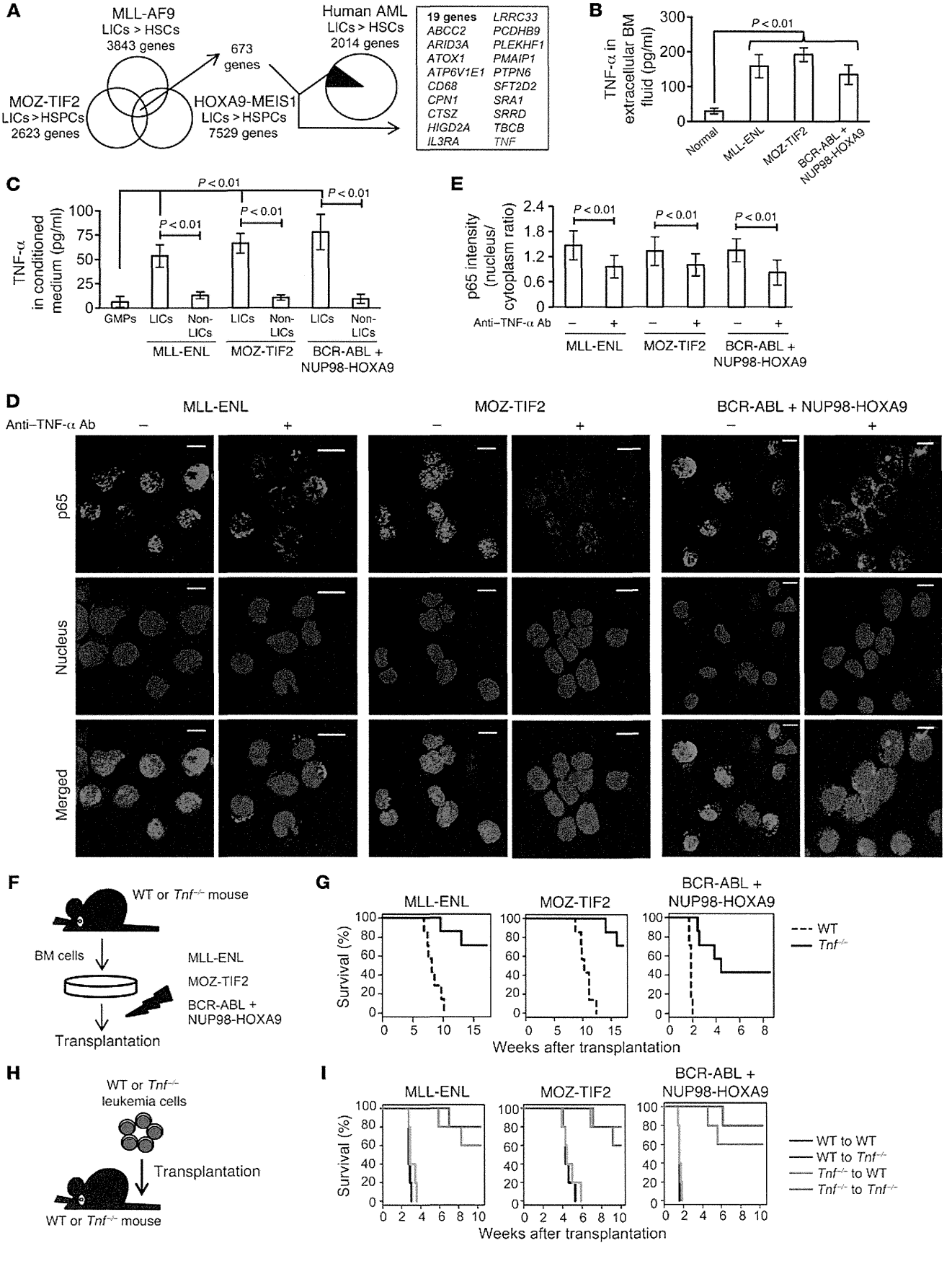

Figure 3
Autocrine TNF-α secretion maintains constitutive NF-κB activity and confers proliferative advantage in LICs. (**A**) Thorough investigation of genes with elevated expression in murine and human LICs compared with that in normal HSPCs in the published gene expression data. (**B**) TNF-α ELISA in extracellular fluid of normal or leukemic BM (n = 4 each). Error bars indicate SD. (**C**) TNF-α secretory ability in LICs compared with that of non-LICs and normal GMPs assessed by ELISA in cultured media (n = 4 each). Error bars indicate SD. (**D**) Immunofluorescence assessment for p65 nuclear translocation in LICs in serum-free culture medium with neutralizing antibody against TNF-α or isotype control. Scale bars: 10 μm. (**E**) Quantification of p65 nuclear translocation of LICs treated with neutralizing antibody against TNF-α or isotype control 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. (**F**) Schematic representation of the experiments. BM cells derived from WT or *Tnf*-knockout mice were transduced with MLL-ENL, 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).

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/NUP98-HOXA9 leukemia cells with IκB-SR. The introduction of IκB-SR significantly decreased a proportion of the cells in the S and G2/M phases of the cell cycle and resulted in a substantial growth delay of those cells in liquid culture (Supplemental Figure 6, C and D). Moreover, leukemia cells with IκB-SR had a reduced colony-forming capacity, while the transduction of IκB-SR into normal HSCs had no significant influence on their colony-forming ability (Figure 4D). Finally, we transplanted leukemia cells with IκB-SR into sublethally irradiated mice and observed a remarkable delay in leukemia progression (Figure 4E). We also confirmed that the developed leukemia cells with IκB-SR had reduced nuclear translocation of p65 compared with that seen in control cells (Supplemental Figure 6E). In contrast, when normal BM cells were transduced with IκB-SR and transplanted into lethally irradiated mice, we observed no significant differences in the reconstitution capacity of the transplanted cells, nor did we find significant differences in peripheral blood cell counts or PBL surface-marker profiles, indicating that NF-κB pathway inhibition exerts a marginal influence on normal hematopoiesis (Supplemental Figure 7, A–C). Collectively, these findings clearly demonstrate that enhanced NF-κB activity in LICs plays a supportive role in leukemia progression and that NF-κB inhibition severely attenuates the proliferative ability of these cells.

To further validate the importance of the NF-κB pathway in leukemia progression, we used BM cells from $Rela^{flox/flox}$ mice (32). We similarly established leukemia cells derived from $Rela^{flox/flox}$ BM cells. Then, the developed leukemia cells were infected with codon-improved Cre recombinase–IRES-GFP (iCre-IRES-GFP) 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-

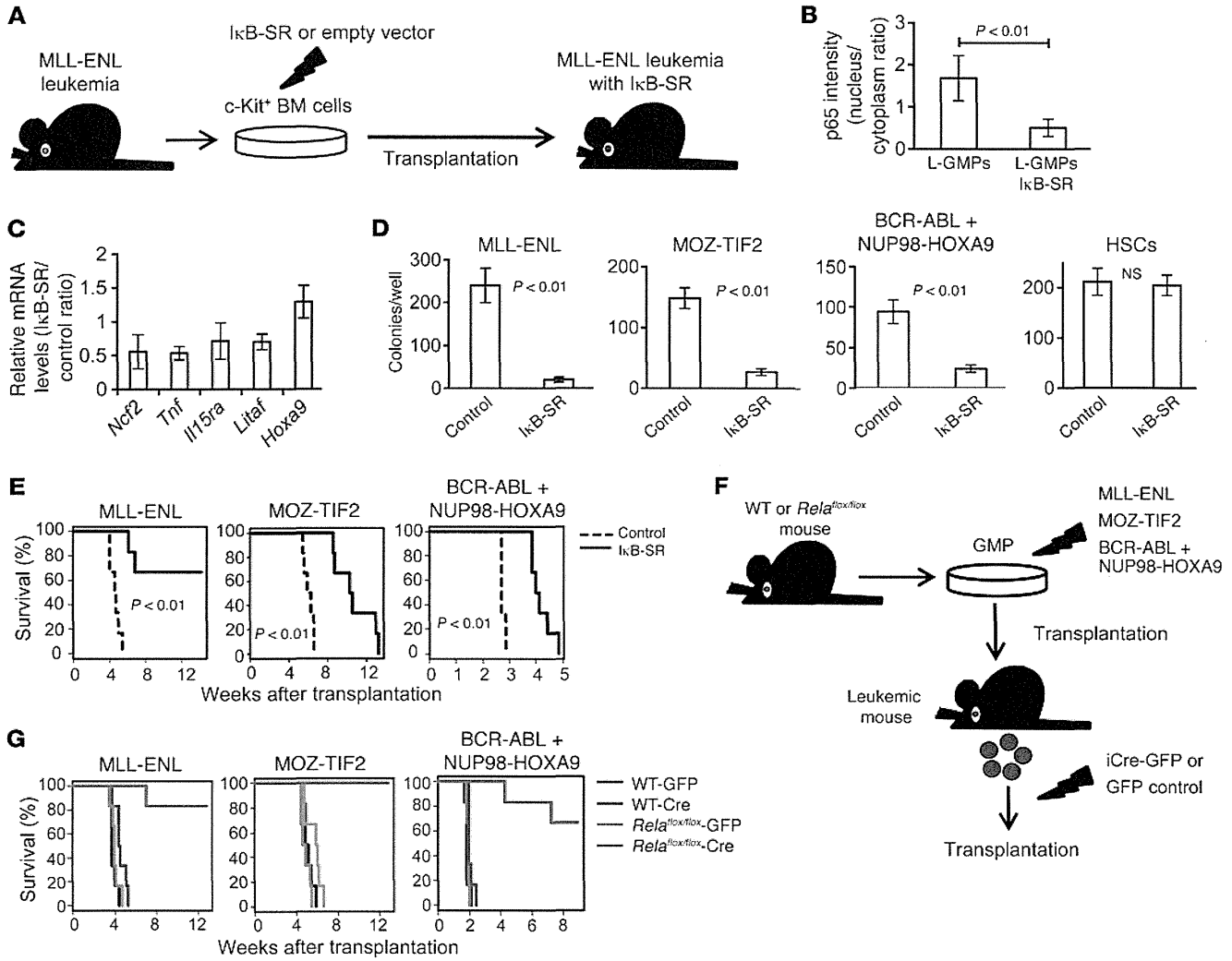

Figure 4

Specific inhibition of NF-κB significantly inhibits leukemia progression in vivo. (**A**) Schematic representation of the following experiments: c-Kit+ BM cells isolated from MLL-ENL leukemic mice were transduced with IκB-SR or control vector and transplanted into sublethally irradiated mice. (**B**) Quantification of p65 nuclear translocation assessed by the mean nucleus/cytoplasm intensity ratio by immunofluorescence staining. More than 50 cells were scored in each specimen, and the average intensity ratio with SD is shown. (**C**) Relative expression profiles of NF-κB target genes in MLL-ENL leukemia cells with or without IκB-SR. The change in *Hoxa9* expression is shown as a control gene not regulated by NF-κB. Error bars indicate SD (n = 3 each). (**D**) CFC assay of leukemia cells and normal HSCS with or without IκB-SR. Cells were seeded at 2,000 cells per well in MLL-ENL or BCR-ABL/NUP98-HOXA9–induced leukemia cells, at 500 cells per well in MOZ-TIF2–induced leukemia cells, and at 1,000 cells per well in normal HSCs (n = 6 in each experiment). (**E**) Survival curves of mice transplanted with MLL-ENL, MOZ-TIF2, and BCR-ABL/NUP98-HOXA9 leukemia cells with or without IκB-SR (n = 6 each). (**F**) Schematic representation of the following experiments: WT or *Rela*$^{flox/flox}$ mice were transduced with MLL-ENL, MOZ-TIF2, or BCR-ABL plus NUP98-HOXA9 and transplanted into sublethally irradiated mice. The developed leukemia cells were transduced with iCre-IRES-GFP or control GFP, and GFP+ cells were secondarily transplanted into mice. (**G**) Survival curves of mice in the experiments shown in **F** (n = 6 each).

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-κB pathway increases LIC frequency in leukemic BM. Given the supportive role of the NF-κB 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-κB 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-κB by holding it in the cytoplasm, its downregulation should function to

enhance NF-κB activity, regardless of the basal proteasome activity. We first confirmed that MLL-ENL leukemia cells with shRNA-mediated knockdown of IκBα (MLL-ENL-IκBα^{KD}) showed decreased IκBα protein levels in the cytoplasm and increased nuclear p65 protein levels, which would indicate that NF-κB signal was enhanced by the reduction of its cytoplasmic inhibitor (Figure 6B). In accordance with this finding, MLL-ENL-IκBα^{KD} cells had a significantly greater ability to secrete TNF-α than did control cells, reflecting an activated NF-κB/TNF-α signaling loop (Figure 6C). We further investigated the phenotype of leukemic mice with MLL-ENL-IκBα^{KD}. Interestingly, the BM of these MLL-ENL-IκBα^{KD} mice showed a marked increase in immature Gr-1^{lo} c-Kit^{hi} 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 significantly different among the leukemia cells, MLL-ENL-IκBα^{KD} 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 IκBα and transplanted them into lethally irradiated 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 positive 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 variation among patients, one that might reflect a heterogeneous distribution 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 parameters 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 Figure 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 antibodies, 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 normal 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 mechanism involved in the maintenance of this pathway activity, which had yet to be elucidated. We found that autocrine TNF-α secretion, with the support of enhanced proteasome activity, contributed 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 recognized as a major regulator bridging the two pathological conditions in different types of malignancies. In most of these malignancies, aberrant activation of the NF-κB pathway derives from inflammatory microenvironments that are mainly created by proinflammatory immune cells such as tumor-infiltrating macrophages, neutrophils, and lymphocytes (34, 35). In this study, however, LICs retained their p65 nuclear translocation even after serum-free culture, suggesting that the constitutive NF-κB activity 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 expression levels that contributed to the maintenance of NF-κB activation in LICs. Conversely, the introduction of IκB-SR markedly suppressed TNF-α expression levels, indicating that NF-κB activity 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 secretory activities in human AML CD34$^+$CD38$^-$ cells and that inhibition of autocrine TNF-α signaling attenuates p65 nuclear translocation. 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 minimal. 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 importance 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