Accelerated Development of Chronic Lymphocytic Leukemia in New Zealand Black Mice Expressing a Low Level of Interferon Regulatory Factor 4*

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A recent genome-wide SNP association study identified IRF4 as a major susceptibility gene for chronic lymphocytic leukemia (CLL). Moreover, the SNPs located in the 3’ UTR of the IRF4 gene have been linked to a down-regulation of IRF4. However, whether a low level of IRF4 is critical for CLL development remains unclear. New Zealand Black (NZB) mice are a naturally occurring, late-onset mouse model of CLL. To examine the role of a reduced level of IRF4 in CLL development, we generated, through breeding, IRF4 heterozygous mutant mice in the NZB background (NZB IRF4\textsuperscript{+/−}). Our results show that CLL development is accelerated dramatically in the NZB IRF4\textsuperscript{+/−} mice. The average onset of CLL in NZB mice is 12 months, but CLL cells can be detected in NZB IRF4\textsuperscript{+/−} mice at 3 months of age. By 5 months of age, 80% of NZB IRF4\textsuperscript{+/−} mice develop CLL. CLL cells are derived from B1 cells in mice. Interestingly, NZB IRF4\textsuperscript{+/−} B1 cells exhibit prolonged survival, accelerated self-renewal, and defects in differentiation. Although NZB IRF4\textsuperscript{+/−} CLL cells are resistant to apoptosis, high levels of IRF4 inhibit their survival. High levels of IRF4 also reduce the survival of MEC-1 human CLL cells. Our analysis further reveals that high levels of IRF4 suppress Akt activity and can do so without the IRF4 DNA binding domain. Thus, our findings reveal a causal relationship between a low level of IRF4 and the development of CLL and establish IRF4 as a novel regulator in the pathogenesis of CLL.

Background: A genome-wide SNP association study has linked low levels of IRF4 with the development of CLL.

Results: Low levels of IRF4 disrupt homeostasis of B1 B cells and promote survival of CLL cell.

Conclusion: Our results demonstrate a causal relationship between low levels of IRF4 and the development of CLL.

Significance: We establish IRF4 as a critical regulator in the pathogenesis of CLL.

A recent genome-wide SNP association study identified IRF4 as a major susceptibility gene for chronic lymphocytic leukemia (CLL). Moreover, the SNPs located in the 3’ UTR of the IRF4 gene have been linked to a down-regulation of IRF4. However, whether a low level of IRF4 is critical for CLL development remains unclear. New Zealand Black (NZB) mice are a naturally occurring, late-onset mouse model of CLL. To examine the role of a reduced level of IRF4 in CLL development, we generated, through breeding, IRF4 heterozygous mutant mice in the NZB background (NZB IRF4\textsuperscript{+/−}). Our results show that CLL development is accelerated dramatically in the NZB IRF4\textsuperscript{+/−} mice. The average onset of CLL in NZB mice is 12 months, but CLL cells can be detected in NZB IRF4\textsuperscript{+/−} mice at 3 months of age. By 5 months of age, 80% of NZB IRF4\textsuperscript{+/−} mice develop CLL. CLL cells are derived from B1 cells in mice. Interestingly, NZB IRF4\textsuperscript{+/−} B1 cells exhibit prolonged survival, accelerated self-renewal, and defects in differentiation. Although NZB IRF4\textsuperscript{+/−} CLL cells are resistant to apoptosis, high levels of IRF4 inhibit their survival. High levels of IRF4 also reduce the survival of MEC-1 human CLL cells. Our analysis further reveals that high levels of IRF4 suppress Akt activity and can do so without the IRF4 DNA binding domain. Thus, our findings reveal a causal relationship between a low level of IRF4 and the development of CLL and establish IRF4 as a novel regulator in the pathogenesis of CLL.

Chronic lymphocytic leukemia (CLL)\textsuperscript{3} accounts for about 30% of all adult leukemias and is the most common hematologic malignancy in the Western world (1). CLL is an incurable B cell malignancy featuring a progressive accumulation of CD5+ B lymphocytes in blood, bone marrow, and lymph node. Many factors have been implicated in the molecular etiology of CLL, including genetic predisposition, autoantigen stimulation, microRNAs, and cytogenetic abnormalities (2). However, the molecular basis of CLL pathogenesis has not been fully elucidated, largely because few genetic abnormalities have been linked conclusively to the development of CLL. Among the common genetic defects in CLL, only the 13q14 deletion has been linked conclusively to the pathogenesis of CLL. The 13q14 deletion is the most common genetic abnormality in CLL and is found in over 50–60% of cases (2). As such, the 13q14 deletion is believed to be a major initiation event in CLL development. Indeed, deletion of a region syntenic to human 13q14 in mice is sufficient to cause late-onset CLL (3). The miR-15a/16-1 cluster was thought initially to be the sole mediator of the tumor-suppressive function in the 13q14 interval (4). However, a recent study indicated that the region adjacent to the miR15a/16-1 cluster may also play an important role in suppressing CLL development (5).

Mouse models of CLL have been useful tools for dissecting the pathogenesis of CLL and for testing therapeutic agents (6). New Zealand Black (NZB) mice are a well described model of spontaneous late-onset CLL (7). Multiple genetic loci have been linked to the development of CLL in NZB mice (8). Interestingly, NZB mice were found to harbor point mutations in the 3’ flanking region of miR16-1 that affect its processing and, hence, its function (8). Restoring the expression of miR16-1 in NZB CLL cells led to enhanced sensitivity to chemotherapeutic agents (9). Similar to other mouse models of CLL, the malignant CLL clones in NZB mice are derived from B1 cells (10). B1 cells are a small B cell subset that normally resides in the peritoneal and pleural cavities. B1 cells are generated from precursors in fetal liver during embryogenesis and from precursors in bone marrow postnatally (11). When generated, the B1 cell population in the peritoneal cavity (PC) is tightly regulated by factors that control their survival and self-renewal. B1 cells play an important role in the host defense against microbial infection and are the major producers of natural antibodies in serum (12). Similar to human CLL cells, B cell receptors (BCR) expressed on B1 cells are polyreactive with a restricted immunoglobulin heavy chain (IgH) repertoire.

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\textsuperscript{3} The abbreviations used are: CLL, chronic lymphocytic leukemia; NZB, New Zealand Black; PC, peritoneal cavity; BCR, B cell receptor; MBL, monoclonal B cell lymphocytosis; BL, blood; BM, bone marrow; IRF, interferon regulatory factor.
Interferon regulatory factor 4 is a critical transcriptional regulator of immune system development and function (13). Previous studies from us and others have shown that IRF4 is essential for B cell development (14, 15). The role of IRF4 in B cell malignancies appears to be developmental-stage-specific. We and others have shown that IRF4 acts as a classical tumor suppressor to prevent pre-B cell transformation (16, 17). However, in plasma cell-derived multiple myeloma, IRF4 behaves as an oncogene to promote the survival of tumor cells (18). A recent genome-wide SNP association study in CLL patients identified IRF4 as a major susceptible gene for CLL (19). Further analysis of the SNPs located in the 3′ UTR of the IRF4 gene has revealed that they are associated with a down-regulation of IRF4 in CLL (20). Low levels of IRF4 in CLL cells were also found to be correlated with a poor prognosis in CLL patients (21). Finally, mutations in the DNA binding domain of the IRF4 gene have also been identified in human CLL patients (22). Although emerging evidence has linked low levels of IRF4 to the development of CLL, it is unclear whether low levels of IRF4 are critical for CLL development. In this report, we examined the effect of reduced levels of IRF4 in CLL development in NZB mice that are heterozygous for an IRF4 knockout allele (NZB IRF4+/−). Our results show that, compared with wild-type NZB mice (NZB IRF4+/+), CLL development was accelerated dramatically in NZB IRF4+/− mice. NZB IRF4+/− CLL cells expressed high levels of Bcl2 and Mcl-1 and were resistant to apoptosis. Additionally, survival, expansion, and differentiation of B1 cells were also defective in the NZB IRF4+/− mice. Finally, we provide evidence that high levels of IRF4 suppressed Akt and promoted apoptosis in CLL cells.

**EXPERIMENTAL PROCEDURES**

**Mice**—IRF4 heterozygous mutant mice (IRF4+/−) have been described previously (23). The Rag2 and common γ chain double-deficient mice (Rag2−/−γ−/−) mice were obtained from Taconic. NZB mice were obtained from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions. Experiments were performed according to guidelines from the National Institutes of Health and with an approved protocol from the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Mice 5–30 weeks of age were used for this study.

**FACS Analysis and Cell Sorting**—Cells were isolated from NZB IRF4+/+ and NZB IRF4+/− mice, preincubated with either 2% rat serum or Fc-Block (2.4G2), and stained with optimal amounts of specific antibodies, either biotinylated or directly fluorochrome-conjugated. All the antibodies were purchased from BD Biosciences. FACS analysis was performed with a FACSCalibur flow cytometer.

**Adoptive Transfer of B1 Cells**—Cells were isolated from PCs of NZB IRF4+/+ and NZB IRF4+/− mice and incubated in tissue culture dishes containing RPMI 1640 medium for 4 h to remove adherent macrophages. The cells in suspension were collected and stained with carboxyfluorescein succinimidyl ester (CFSE) dye and injected into the PCs of non-irradiated Rag2−/−γ−/− host mice. 10⁵ cells were used for each injection, and three host mice were used for each group. 10 days later, the transplanted cells were isolated for FACS analysis.

Ki67 Staining of CLL Cells—Cells isolated from blood, lymph node, and spleen of either NZB IRF4+/+ and NZB IRF4+/− mice were stained with antibodies against CD5, IgM, and B220. After fixation, the Ki67-positive cells were measured with a kit from BD Biosciences. The percentages of Ki67-positive cells were revealed by FACS analysis.

**In Vivo BrdU Labeling**—The in vivo BrdU labeling assay was performed as described before (24). Mice were injected intraperitoneally with 6 mg/ml BrdU (Sigma-Aldrich), and 12 h later, the cells were isolated for analysis. Three mice from each group were used for this assay. Cells from blood, bone marrow, lymph node, and spleen were stained with antibodies against CD5, IgM, and CD19. After fixation, the incorporated BrdU was revealed with a BrdU flow kit (BD Biosciences). The percentages of BrdU-positive cells were detected by FACS.

**Assays to Detect Apoptosis (TUNEL, Caspase 3, and Annexin V)**—The apoptosis status of CLL and control cells in mice was examined with a TUNEL assay. The TUNEL assay was conducted as described previously (17). The cells were isolated and stained with surface antibodies (CD5 and IgM). The TUNEL positive cells were revealed with an APO-direct kit (BD Biosciences). Activated caspase 3 and annexin V staining were also used to detect apoptotic cells. In this case, the assays were carried out with kits from BD Biosciences.

**Assay to Measure Phospho-Akt**—MEC-1 cells were fixed in 2% paraformaldehyde for 10 min and permeabilized in 100% methanol for 30 min. The permeabilized cells were incubated with anti-phospho-Akt Ser-473 antibody (Alexa Fluor 488 conjugate, Cell Signaling Technology) for 1 h at room temperature. After washing, the intracellular phospho-Akt activity was examined by FACS.

**Assay to Measure miR15a/16-1**—Total RNA was extracted from the cells with a microRNA isolation kit (Ambion). Total RNA was converted to cDNA using a TaqMan microRNA reverse transcription kit and TaqMan RT primers (ABI). For microRNA quantification, TaqMan microRNA assays (ABI) were used according to the protocol of the manufacturer. Expression levels were normalized to the U6 snRNA.

**Transfection of CLL Cells in Vitro**—CLL cells were isolated from spleens of NZB IRF4+/− mice and cultivated on top of the S17 stromal layer in medium containing RPMI 1640 with 10% FBS. To reconstitute expression of IRF4, NZB IRF4+/− CLL cells were mixed with either control vector (MigR1) or IRF4-expressing vector (MigIRF4). 10 × 10⁶ CLL cells and 20 μg of plasmid were used for each transfection. The transfection was carried out in a Nucleofector™ (Lonza) with Solution V using program G-016. The transfected cells were analyzed 48 h later. For transfection of human MEC-1 cells, 2 × 10⁶ cells and 20 μg of plasmid were used for each transfection. The condition for MEC-1 transfection was Solution V and program X-001. The expression plasmids MigR1, MigIRF4, and MigIRF8 have been described before (25). MigIRF4Del contains a truncated version of IRF4 lacking the N-terminal DNA binding domain (the beginning 150 amino acids).

**Measurement of Calcium Influx**—Splenocytes were isolated from NZB IRF4+/+ and NZB IRF4+/− mice and stained with antibodies against CD5 and B220. After washing, the stained cells were incubated with 1 μM of Indo-1 AM (Molecular
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Probes) for 30 min at 37 °C in RPMI 1640 medium containing 3% FBS. The calcium influx of loaded cells was analyzed with a LSR II flow cytometer. The base-line emission of the fluorescence ratio (405/525 nm) of CLL or B1 cells was collected for 1 min. Then, anti-μ antibody (Jackson ImmunoResearch Laboratories Inc.) at 5 μg/ml was added, and the fluorescence ratio was recorded for another 4 min. The increase in the fluorescence ratio was used to reflect the intensity of calcium mobilization upon BCR activation. The data were analyzed with Flowjo software.

Western Blot Analysis—Splenic B cells were isolated via negative selection. Briefly, the splenocytes were first incubated with biotinylated antibodies against CD3, CD4, CD8, Ter119, DX5, and Gr-1. After washing, the cells were incubated with streptavidin microbeads. The negatively selected B cells were incubated with 10 μg/ml goat F(ab)2 anti-mouse IgM (Jackson ImmunoResearch Laboratories Inc.) at 37 °C for 5 min. The cells were lysed, and proteins were separated by SDS-PAGE gel. The membranes were incubated with the indicated antibodies, and the signals were revealed with an ECL detection system (Pierce). The antibodies against Akt, phospho-AktSer473 Erk, phospho-Erk, Tcl-1, Bcl-2, Bcl-xl, and Mcl-1 were obtained by Santa Cruz Biotechnology.

RESULTS

Precocious CLL Development in NZB Mice Expressing Low Levels of IRF4—We used IRF4-deficient mice (C57B6) to study the role of IRF4 in B cell development and function (15). However, neither IRF4+/− nor IRF4+/− mice developed CLL (Fig. 1A and data not shown). This observation indicates that low levels of IRF4 are insufficient by itself to cause CLL in mice. To determine whether reduced levels of IRF4 could synergize with other genetic defects to accelerate CLL development, we backcrossed the IRF4-deficient mice from the C57B6 background onto the NZB background for at least seven generations to generate NZB IRF4+/− mice. To monitor CLL development, we collected blood monthly from saphenous veins of NZB IRF4+/− mice and NZB IRF4+/+ control mice. Ten mice from each group were used. The appearance and percentage of a monoclonal B220lowCD5+ in the peripheral blood mononuclear cells were used for the initial CLL diagnosis. The diagnostic criteria for CLL in NZB mice were a monoclonal population of B220lowCD5+ cells constituting over 20% of peripheral blood mononuclear cells. If the percentage of the B220lowCD5+ clone was under 20% of the peripheral blood mononuclear cells, a diagnosis of monoclonal B cell lymphocytosis (MBL) was made.

The latency to onset of CLL in NZB IRF4+/− mice is typically around 12 months. Interestingly, two NZB IRF4+/− mice developed CLL after just three months (Fig. 1A). After five months, eight NZB IRF4+/− mice developed CLL, whereas the remaining two developed MBL. The NZB IRF4+/− mice with MBL all progressed into CLL within 10 months. In contrast, none of the mice in the NZB IRF4+/− control group developed CLL or MBL within 10 months. Only three of 10 NZB IRF4+/− mice developed CLL/MBL within 13 months (Fig. 1A). To characterize CLL development in NZB IRF4+/− mice, we analyzed CLL cells in PC, blood (BL), bone marrow (BM), and spleen (SPL) of five-month-old NZB IRF4+/− by FACS (Fig. 1B). The control NZB IRF4+/− mice were also examined. B1 cells normally reside in the PC and, like CLL cells, are B220lowCD5− cells. However, the presence of a significant amount of B220lowCD5+ CLL cells was also detected in the blood, bone marrow, and spleen of NZB IRF4+/− mice. In NZB IRF4+/− mice, 80% of splenocytes were B220lowCD5+ CLL cells (Fig. 1B). The infiltrations of CLL cells in spleens of NZB IRF4+/− mice caused massive splenomegaly where an average accumulation of 3.6 ± 1.4 × 109 CLL cells was observed. Besides in the spleen, CLL cells were also detected in the lymph node of NZB IRF4+/− mice and caused moderate lymph adenopathy (data not shown). Together, our results show that CLL development is accelerated dramatically in NZB IRF4+/− mice.

Phenotypical and Histological Characterization of NZB IRF4+/− CLL Cells—To further characterize CLL cells in NZB IRF4+/− mice, we stained the NZB IRF4+/− CLL cells with a panel of antibodies against B220, IgM, IgD, CD21, CD23, and CD43. IgD is a mature B cell marker that is normally expressed at higher levels on follicular B cells but at low levels on B1 cells. CD21 and CD23 are markers for follicular B cells, but they are expressed at low levels on B1 cells. CD43 is a B cell activation marker and is expressed at high levels on B1 cells but not on follicular B cells. Our result shows that NZB IRF4+/− CLL cells were B220lowIgMhi IgDlowCD21−CD23−CD43+, a surface phenotype that resembles B1 cells, from which they were derived (Fig. 2A). We also performed a histological analysis of spleens of NZB IRF4+/− mice. Histological examination of the
In contrast, 15.3% of BRD4-positive NZB IRF4+/+ cells were stained positively for Ki-67, indicating that the expansion of NZB IRF4+/+ cells occurs predominantly in the spleen and are resistant to apoptosis.

NZB IRF4+/+ CLL Cells Expand Predominantly in the Spleen and Are Resistant to Apoptosis—The expansion of human CLL cells occurs in proliferation centers found predominantly in bone marrow and lymph node (26). Because CLL cells can be detected in several lymphoid organs and tissues, we wanted to identify the locations where expansion of NZB IRF4+/+ CLL cells occurs. To address this question, we measured the expression of Ki-67 in CLL cells isolated from blood, bone marrow, lymph nodes, and spleens of NZB IRF4+/+ mice (Fig. 3A). Only 3 ± 0.8% NZB IRF4+/+ CLL cells in blood were found to express Ki-67. Similarly, only 4.2 ± 1.4% and 5.1 ± 1.9% of CLL cells were Ki-67-positive in bone marrow and lymph nodes. These results indicate that blood, bone marrow, and lymph nodes are not the major sites of proliferation for NZB IRF4+/+ CLL cells. In contrast, 15.3 ± 4.1% of splenic CLL cells in NZB IRF4+/+ mice was stained positively for Ki-67, indicating that the expansion of NZB IRF4+/+ CLL cells occurs predominantly in the spleen. This finding is consistent with the result of a BrdU pulse-labeling analysis that shows that 9% of CLL cells in the spleen were BrdU-positive, whereas percentages of BrdU+ CLL cells in BL, BM, and lymph nodes were only 3% or lower (Fig. 3B). We further analyzed the apoptotic status of NZB IRF4+/+ CLL cells in blood and spleen and compared them with B cells in NZB IRF4+/− mice (Fig. 3C). The percentages of TUNEL-positive cells in both blood and spleen were found to be significantly lower in the NZB IRF4+/− mice than in the NZB IRF4+/+ control mice. Together, these results indicate that NZB IRF4+/+ CLL cells proliferate predominantly in the spleen and are resistant to apoptosis.

NZB IRF4+/+ CLL Cells Show Hyperresponsiveness to BCR Stimulation—BCR signaling is critical for the survival and expansion of CLL cells (27). To study BCR signaling in NZB IRF4+/+ CLL cells, we examined the calcium influx triggered by BCR cross-linking in isolated NZB IRF4+/+ CLL cells. The sple-
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![B and T cell receptor signaling](https://jbc.asm.org/lookup/doi/10.1074/jbc.M113.491852)

**FIGURE 4.** NZB IRF4−/− CLL cells are hyperresponsive to BCR stimulation. A, isolated splenocytes from NZB IRF4−/− and NZB IRF4+/+ mice were stained with antibodies against B220 and CD5. The stained cells were incubated with Indo-1 Am for 20 min at 37 °C. The calcium influx induced by 5 μg/ml anti-IgM antibodies was recorded by a LSR II flow cytometer. The calcium influx by CLL cells and B1 cells was overlaid with Flowjo software. Dark line, NZB IRF4−/− B1 cells; light line, NZB IRF4−/−/H11001 B1 cells. B, splenic CLL cells were isolated from NZB IRF4−/− mice via negative selection. The isolated cells were stimulated with anti-IgM antibody at 10 μg/ml for 5 min. Splenic B cells isolated from NZB IRF4−/− mice were analyzed as a control. Western blot analysis was performed to detect phospho-Erk and Akt. C, CLL cells were isolated from spleens of NZB IRF4−/− mice and plated on top of 517 stromal cells in RPMI 1640 medium containing 10% FBS. MigIRF4 and MigR1 vectors were transfected into cultivated CLL cells using a Nucleofector™. Two days after transfection, the cells were stained with Indo-1 Am and stimulated with 5 μg/ml anti-IgM. Ca2+ influx in GFP+ cells was examined. MigR1- and MigIRF4-transfected cells are indicated by arrows.

Molecular Characterization of NZB IRF4−/−/H11001 CLL Cells—To further characterize NZB IRF4−/−/H11001 CLL cells, we examined some common molecular signatures that are associated with human CLL cells, including abnormal expression of Bcl-2, Mcl-1, T cell leukemia/lymphoma 1 (Tcl1), and miR15a/16-1 microRNAs. Splenic CLL cells were isolated from six NZB IRF4−/−/H11001 mice, and the expression of the Bcl-2 family members Bcl-2, Bcl-xl and Mcl-1 was examined by Western blot analysis. Splenic B cells from NZB IRF4−/−/H11001 mice were used as a control. Similar to human CLL cells, the expression of Bcl-2 and Mcl-1 was elevated significantly in all six CLL samples (Fig. 5A). In contrast, the expression of Bcl-xl was only increased moderately in some but not all samples. The expression of Tcl1 can be detected in 90% human CLL cases and is found to be overexpressed in patients with aggressive CLL (28). Moreover, mice engineered to overexpress the Tcl1 oncogene in B cells (EμTcl1) develop late-onset, aggressive CLL (29). It has been shown that the Tcl1 oncogene can promote the survival of CLL cells by directly interacting with Akt (30, 31). However, except for CLL cells isolated from EμTcl1 transgenic mice, the expression of Tcl1 cannot be detected in samples derived from NZB IRF4−/−/H11001 mice, indicating that the expression of Tcl1 is very low in those samples. Additionally, total RNA was isolated from NZB IRF4−/−/H11001 CLL cells, and the expression of miR15a and 16-1 were measured by real-time PCR. RNA isolated from splenic B cells of NZB IRF4−/−/H11001 mice was examined and used as a control. Among the four CLL samples examined, the expression levels of miR15a/16-1 were comparable with that of the control (Fig. 5B). In summary, our results show that the expression of Bcl-2 and Mcl-1 is elevated significantly in NZB IRF4−/−/H11001 CLL cells. However, the expression of Tcl1 and miR15a/16-1 is not deregulated in these cells.
**Molecular characterization of NZB IRF4−/− CLL cells**

**A**

NZBIRF4+/− CLL cells were isolated from six NZB IRF4−/− mice via negative selection and lysed for Western blot analysis with the indicated antibodies. Splenic B cells from NZB IRF4−/− mice were also isolated and analyzed as controls (Con). Additionally, splenic CL cells from EpTcl1 transgenic mice were used as a positive control for Tcl-1 expression. The numbers below each lane indicate the fold change in comparison to the control. The intensity of each protein was normalized initially to β-actin. B, total RNA was also extracted from the isolated cells. Real-time TaqMan PCR to detect expression of miR15a/16-1 was done using a kit from Applied Biosystems. The data were normalized to U6 snRNA and were expressed as fold change in comparison with the control.

**B1 Cells in NZB IRF4+/− Mice Show Defects in Survival, Expansion, and Differentiation**—Because NZB IRF4+/− mice developed early-onset CLL, we wanted to examine the impact of a reduced level of IRF4 on normal B1 cell development and function. For this study, we used 5- to 6-week-old NZB IRF4+/− mice before they developed CLL. Compared with NZB IRF4+/+ mice, the number of B1 cells in NZB IRF4+/− mice increased significantly in PCs. The number of B1 cells were 8.2 ± 3.8 × 10^6 in NZB IRF4+/− mice but only 4.1 ± 2.1 × 10^6 in NZB IRF4+/− mice (Fig. 6A). This is indicative of defects in B1 cell homeostasis in NZB IRF4−/− mice. We further analyzed the survival and expansion of B1 cells in IRF4+/− NZB mice. For the survival analysis, we measured spontaneous apoptosis of B1 cells in vitro. B1 cells were isolated from PCs of NZB IRF4+/+ and NZB IRF4−/− mice and cultivated in vitro for 24 h. The apoptotic cells were revealed by propidium iodide staining. Although 15 ± 3.2% NZB IRF4+/− B1 cells underwent apoptosis, only 5.8 ± 1.7% NZB IRF4−/− B1 cells were apoptotic, indicating that NZB IRF4+/− B1 cells are more resistant to apoptosis (Fig. 6B). B1 cell homeostasis in the IRF4 is regulated by a balance between survival and self-renewal. To measure B1 cell expansion (self-renewal), we isolated B1 cells from the PCs of NZB IRF4+/− and NZB IRF4−/− mice. The isolated B1 cells were stained with CFSE dye and transplanted into the PCs of Rag2−/−γ−/− deficient host mice. Ten days later, the transplanted B1 cells were isolated and analyzed by FACS. On the basis of the dilution of CFSE dye, NZB IRF4+/− B1 cells expanded at a much faster rate in host mice than in NZB IRF4+/− B1 cells (Fig. 6C).

Upon antigen encounter, B1 cells can differentiate into short-lived plasma cells. To determine whether reduced levels of IRF4 affect B1 cell differentiation, B1 cells were isolated from NZB IRF4+/− and NZB IRF4−/− mice and incubated with LPS. Three days later, differentiated plasma cells (CD138+) were revealed by FACS. Although 26% of LPS-treated NZB IRF4+/− B1 cells were CD138+ plasma cells, only 14% of LPS-treated NZB IRF4−/− B1 cells were CD138+ plasma cells (Fig. 6D). This result indicates that NZB IRF4+/− B1 cells have defects in differentiation. Taken together, our results show that there are defects in the homeostasis of NZB IRF4−/− B1 cells, resulting in prolonged survival, enhanced self-renewal, and decreased differentiation.

**High Levels of IRF4 Expression Inhibit Survival of NZB IRF4+/− CLL Cells**—The expression level of IRF4 is presumably reduced by 50% in NZB IRF4+/− mice. To confirm this, we measured the expression levels of IRF4 in isolated NZB IRF4+/− CLL clones. Compared with NZB IRF4+/+ B1 cells, the expression levels of IRF4 were reduced by 50–60% in CLL clones isolated from NZB IRF4+/− mice (Fig. 7A). This result confirmed that IRF4 is expressed at reduced levels in NZB IRF4+/− CLL cells. Our result shows that NZB IRF4+/− CLL cells are resistant to apoptosis. To determine the effect of expression levels of IRF4 on the survival of NZB IRF4+/− CLL cells, we raised the level of IRF4 in NZB IRF4+/− CLL cells via a Nucleofector (described in Fig. 4C). Although 3.9 ± 1.1% cells underwent apoptosis in control vector-transfected CLL cells, 8.8 ± 3.2% of MigIRF4-transfected CLL cells were apoptotic (Fig. 7B). We further sorted GFP+ cells from transduced cells and measured the expression levels of IRF4 transcripts by real-time PCR. Our result shows that the expression level of the IRF4 transcript was about 3-fold higher in MigIRF4 transduced cells than in NZB IRF4+/− B1 cells. In summary, our finding indicates that survival of NZB IRF4+/− CLL cells is inversely correlated with the expression level of IRF4.

**High Levels of IRF4 Expression Suppress Akt Activity and Promote Apoptosis in Human CLL Cells**—A previous study has shown that low levels of IRF4 are associated with aggressive CLL in human patients (21). We wanted to examine the effect of the IRF4 expression level on human CLL cells. MEC-1 is the most widely used human CLL cell line (32). The expression level of IRF4 is significantly lower in MEC-1 cells than in the multiple myeloma cell lines H929, U266, and RPMI8226 (Fig. 8A). To examine the effect of high levels of IRF4 on CLL cells, we transfected MEC-1 cells with the MigR1 and MigIRF4 expression plasmids. Additionally, MEC-1 cells were also transfected with MigIRF8 and MigIRF4Del (IRF4 without its N-terminal DNA binding domain). IRF8 is closely related to IRF4 in terms of sequence homology and function (33). Our previous studies have shown that IRF4 and IRF8 function redundantly to control pre-B cell development (25). Two days after transfection, the cells were stained with annexin V and analyzed by FACS. Although only 4% of MigR1-transfected cells were annexin V-positive, 18% of MigIRF4-transfected cell underwent apoptosis, indicating that a high level of IRF4 promotes apoptosis (Fig. 8A). Interestingly, 15% of IRF4Del-transduced
cells were also stained positive for annexin V, indicating that IRF4 does not need its DNA binding domain to promote apoptosis in MEC-1 cells. Unlike IRF4, a high level of IRF8 did not affect the survival of MEC-1 cells. Because the target gene and GFP are translated from the same mRNA transcript through an internal ribosome entry site, the expression levels of GFP can be used as an indicator of target gene expression. Among the different plasmids, the percentages of GFP-positive cells and the intensity of GFP appear to be inversely correlated with their effect on MEC-1 cells. MigR1- and IRF8-transduced cells expressed the highest levels of GFP, whereas IRF4- and IRF4Del-transduced cells expressed the lowest levels (Fig. 8C).

It has been shown that the PI3K/Akt pathway is critical for the survival of human CLL cells (34). Akt is activated by phosphorylation at multiple sites, and among them, Akt phosphorylation at Ser-473 is required for its activation. A recent study has shown that Akt is constitutively phosphorylated (Ser-473) in MEC-1 cells and that inhibition of Akt phosphorylation dramatically reduces the survival of MEC-1 cells (35). We wanted to examine the effect of high levels of IRF4 on Akt activation. To this end, we measured phosphorylated Akt (Ser-473) in MEC-1 cells transfected with MigR1, IRF4, and IRF4Del. MEC-1 cells were also treated with the PI3k inhibitor LY294002 and analyzed as a control. As shown in Fig. 8D, LY294002 treatment decreased the percentage of phospho-Akt-expressing cells from 83% in untreated cells to 29% in treated cells. Additionally, LY294002 treatment also led to enhanced cell apoptosis (data not shown). Although 70% of phospho-Akt-expressing cells were found in MigR1-transfected cells, the percentages of phospho-Akt-expressing cells in cells transfected with IRF4 and IRF4Del were only 24 and 35%, respectively (Fig. 8D). The suppressive effect of IRF4 and IRF4del on Akt phosphorylation was confirmed by statistical analysis of three independent experiments (Fig. 8E). In summary, our results show that high levels of IRF4 suppress Akt and promote apoptosis in human CLL cells. Moreover, IRF4 can do so without its DNA binding domain, albeit less efficiently.

**DISCUSSION**

A previous genome-wide SNP association study has linked low levels of IRF4 with the development of CLL (19). In this study, we demonstrate for the first time a causal relationship between a reduced level of IRF4 and the development of CLL. We used IRF4 heterozygous mutant NZB mice to mimic the effect of germ line SNP variants in the IRF4 gene and the associated low levels of IRF4 in CLL patients. Our results show that CLL development was accelerated dramatically in NZB IRF4+/− mice. The average onset of CLL in NZB mice is around 12 months, but CLL cells could be detected in NZB IRF4+/− mice at 3 months of age. By 5 months of age, 80% NZB IRF4+/− mice developed CLL. In contrast to what is seen in humans, our analysis reveals that the spleen, but not lymph nodes and bone marrow, is the major site of the expansion for NZB IRF4+/− CLL cells. The expansion of human CLL cells occurs in the proliferation center found predominantly in lymph nodes and bone marrow. It is generally believed that the survival and expansion of CLL cells in the proliferation center are dependent on the presence of antigen in a unique microenvironment that consists of nurse-like cells, stromal cells, follicular dendritic cells, and helper T cells (26, 36). It is likely that the splenic microenvironment provides the much-needed prosurvival/proliferation signals in this mouse model. Human CLL cells, like murine B1 cells, possess polyreactive BCRs that recognize self-antigen and microbial antigen, and chronic autoimmune stimulation is believed to play a critical role in the development and progression of CLL (36, 37). Our result shows that NZB...
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IRF4+/− B cells are hyperresponsive to BCR stimulation, a property that would render them more sensitive to autoantigen stimulation in vivo and, thus, confer a survival advantage for the cells. However, reconstitution of IRF4 expression in NZB IRF4+/− CLl clones did not attenuate BCR signaling, suggesting that BCR signaling is not directly regulated by expression levels of IRF4.

Our results show that NZB IRF4+/− CLL cells express high levels of Bcl-2 and Mcl-1. Human CLL cells are known to overexpress Bcl-2 and Mcl-1 as well as other members of prosurvival Bcl-2 family proteins. Among the Bcl-2 family members, Mcl-1 is the major prosurvival factor for CLL cells (34). Clinically, Mcl-1 is found to be better than other Bcl-2 family members at predicting prognosis and clinical behavior of CLL patients (38). Elevated levels of Bcl-2 in CLL cells have been attributed to promoter hypomethylation and loss of the negative regulators miR15a/16 (39, 40). Our results show that, in NZB IRF4+/− CLL cells, miR15a/16-1 are expressed at levels that are comparable with those in NZB IRF4+/− B cells, indicating that an elevated level of Bcl-2 is not the result of deregulated expression of miR15a/16-1.

Our results show that IRF4 is a critical regulator of B1 cell homeostasis. Although the cellular origin of human CLL remains unclear, CLL cells are believed to be derived from B1 cells in mice (10, 29, 41). Our results show that B1 cells in NZB IRF4+/− mice exhibit prolonged survival and enhanced self-renewal, resulting in a dramatic expansion of B1 cells. Upon antigen encounter, B1 cells can differentiate into short-lived antibody-producing plasma cells. However, NZB IRF4+/− B cells are defective in plasma cell differentiation. It has been demonstrated that IRF4 is required for plasma cell differentiation (42, 43). Therefore, the defects in differentiation of NZB IRF4+/− B cells are most likely because of an insufficient amount of IRF4 in those cells. Taken together, these observations support a scenario where prolonged survival, enhanced self-renewal, and expansion, together with defects in differentiation in NZB IRF4+/− B cells, lead to an expansion of precursor CLL cells and render them more susceptible to subsequent transformation events.

Besides regulating the survival and expansion of precursor CLL cells (B1), our results also show that IRF4 directly controls the survival of CLL cells. A rise in the levels of IRF4 in NZB IRF4+/− CLL cells promotes apoptosis. Moreover, a rise in the levels of IRF4 in MEC-1 human CLL cells reduces their survival as well. In contrast, our result shows that an increase in the levels of IRF8 has no effect on the survival of CLL cells. A recent genome-wide SNP association study identified IRF8 as a novel susceptibility gene for CLL (44). However, in this case, the SNPs located in the IRF8 gene are linked to high levels of IRF8 in CLL patients (44). Our previous study has shown that IRF8 and IRF4 function in a redundant fashion to control the early stage of B cell development (15). However, it appears that IRF4 and IRF8 play opposite roles in the development of CLL. Further analysis of the effect of IRF4 reveals that high levels of IRF4 suppress Akt activity. Akt activity is regulated by PI3K downstream of BCR signaling and that it controls the expression of several proteins that are critical for the survival of CLL cells. It has been shown that Akt can increase the expression of Mcl-1 by stabilizing Mcl-1 protein and preventing its degradation (45, 46). The Akt/Mcl-1 pathway has been shown to be critical for the survival of CLL cells (34). Besides inducing Mcl-1 expression, Akt has been shown to phosphorylate and inactivate the proapoptotic proteins Bcl-2 associated death promoter (BAD) and caspase 9 (47, 48).

Because high levels of IRF4 in NZB IRF4+/− CLL cells did not attenuate BCR signaling, the suppressive effect of IRF4 on Akt is not likely to be a result of weakened BCR signaling in CLL cells. How Akt activity is suppressed by IRF4 is unclear. Intriguingly, our results show that truncated IRF4 without its DNA binding domain can still suppress Akt and promote apoptosis in CLL cells. These findings indicate that high levels of IRF4 inhibit the survival of CLL cells through means that are independent of its role as a transcriptional regulator. It is worth noting that our finding is in line with a recent study that shows that IRF4, without its DNA binding domain, can still suppress BCR/ABL (Abelson tyrosine kinase) oncogene-induced myeloid leukemia (49). Their analysis further shows that it is the C-terminal IRF
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**FIGURE 8.** High levels of IRF4 inhibit AKT and promote apoptosis in human CLL cells. A, MEC-1 human CLL cells, H929, U266, and RPMI8226 multiple myeloma cells were lysed for Western blot analysis to detect IRF4 expression. B, MEC-1 cells were transfected with MigR1, MigIRF4, MigIRF4Del, or MigIRF8 expression plasmids. Two days later, the transfected cells were stained with annexin V antibody and analyzed by FACS. The percentages of GFP and annexin V double-positive apoptotic cells were plotted with Graphpad Prism software. The values are mean ± S.D. of three independent experiments. ***, p < 0.01. C, the percentage of GFP-positive MEC-1 cells transfected with MigR1, MigIRF4, MigIRF4Del, and MigIRF8. Forward scatter (FSC). The numbers indicate the percentage of GFP + cells. D, MEC-1 cells were transfected with MigR1, MigIRF4, and MigIRF4Del, and 48 h after transfection, phospho-Akt and GFP double-positive cells were measured by FACS in each group. Additionally, MEC-1 cells were treated with the PI3K inhibitor LY294002 (50 μM). After 2 days, intracellular phospho-Akt was analyzed by FACS. The untreated cells were analyzed as a control. The numbers are percentages of phospho-Akt-positive cells. E, means ± S.D. of three independent experiments described above. ***, p < 0.01.

association domain that is critical for the tumor-suppressive activity of IRF4. Because the DNA binding domain of IRF4 contains a nuclear localization signal, the truncated IRF4 is localized predominantly in the cytosol (49, 50). How cytosolic IRF4 suppresses Akt activity remains unclear. Interestingly, it has been shown that cytosolic IRF4 can interact directly with MyD88 to inhibit its activity (51).

SNPs in the 3’UTR of the IRF4 gene were identified as a risk allele for both sporadic and familial CLL, and at least one copy of the risk allele is present in over 86% of CLL cases (52). The prevalence of the risk allele in CLL patient indicates that it may play an important role in the initiation of CLL. Indeed, our finding that NZB IRF4+/− mice develop early-onset CLL supports this assertion. Although the molecular mechanism through which IRF4 suppresses CLL development remains to be further elucidated, our findings presented here demonstrate that a low level of IRF4 leads to deregulated homeostasis of precursor CLL cells (B1) and prolonged survival of CLL cells, thereby promoting CLL development.

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