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Defining the role of Interferon Regulatory Factor 4 in Chronic Lymphocytic Leukemia.

Vipul Shukla
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**DEFINING THE ROLE OF INTERFERON REGULATORY FACTOR 4 IN CHRONIC LYMPHOCYTIC
LEUKEMIA**

by

VIPUL SHUKLA

A DISSERTATION

Presented to the Faculty of the
University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

**GENETICS, CELL BIOLOGY AND ANATOMY
GRADUATE PROGRAM**

Under the supervision of Professor **Runqing Lu***

**University of Nebraska Medical Center
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April, 2016

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ABSTRACT
DEFINING THE ROLE OF INTERFERON REGULATORY FACTOR 4 IN CHRONIC
LYMPHOCYTIC LEUKEMIA
VIPUL SHUKLA Ph.D.
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SUPERVISOR: RUNQING LU, Ph.D.

Chronic Lymphocytic Leukemia (CLL) represents the most common adult leukemia in the Western hemisphere. Despite considerable progress in our current understanding of CLL, this disease remains incurable and the molecular events underlying the complex pathogenesis of CLL are not fully elucidated. Interferon Regulatory Factor 4 (IRF4) belongs to the IRF superfamily of transcription factors that has been shown to play critical roles at multiple stages of B cell development. Interestingly, a Genome Wide Association Study identified Single Nucleotide Polymorphism (SNP) mediated IRF4 down regulation, as a major predisposing genetic event during the development of CLL. However, whether low levels of IRF4 are causally related to CLL development was unclear. In our studies here, we demonstrated that IRF4 deficient mice expressing immunoglobulin heavy chain Vh11 (IRF4^{-/-}Vh11) developed spontaneous CLL with complete penetrance. Additionally, we also show that low levels of IRF4 dramatically accelerates CLL development in the New Zealand Black (NZB) mouse model of CLL. Together, these studies establish a causal role for IRF4 in the development of CLL. Furthermore, we used the IRF4^{-/-}Vh11 as a novel mouse model to CLL to define the molecular mechanism through which IRF4 suppresses CLL development. Our studies identified hyperactivation of Notch signaling pathway as a common feature of IRF4^{-/-}Vh11 CLL cells. Intriguingly, deregulation of Notch signaling pathway has been identified as one of the most recurrent molecular anomalies in the pathogenesis of CLL. Yet, the role of Notch signaling as well as its regulation during CLL development remained unclear. Our studies further reveal that Notch signaling promotes survival and expansion of CLL cells and their precursors and is indispensable for CLL development in the IRF4^{-/-}Vh11 mice. Moreover, we identify E3 ubiquitin ligase Nedd4, which target Notch for degradation, as a direct target of IRF4 in CLL cells and their precursors. Collectively, our studies here establish a causal role for low levels of IRF4 in the development of CLL. These studies provide the first *in vivo* evidence for an essential role of Notch signaling in the development of CLL and establish IRF4 as a critical regulator of Notch signaling during CLL development.

LIST OF ABBREVIATIONS

CLL: Chronic Lymphocytic Leukemia

IRF4: Interferon Regulatory Factor 4

IRF8: Interferon Regulatory factor 8

NZB: New Zealand Black

Vh11: Immunoglobulin variable heavy chain gene family 11

BCR: B cell Receptor

GWAS: Genome Wide Association Study

SNP: Single Nucleotide Polymorphism

ISRE: Interferon Stimulated Response Element

EICE: Ets-Interferon Composite Element

MZ: Marginal Zone

FO: Follicular

Nedd4: Neural precursor cell expressed developmentally down-regulated protein 4

WGS: Whole Genome Sequencing

RNA-Seq: RNA sequencing

ChIP-Seq: Chromatin Immunoprecipitation Sequencing

CHAPTER 1

INTRODUCTION

Some of the material included in this chapter has been previously published: *Shukla V and *Lu R. "IRF4 and IRF8: Governing the virtues of B lymphocytes." (Review) *Front Biol.* **2014** Aug; 9(4): 269–282. doi: 10.1007/s11515-014-1318-y. *co-corresponding authors

1.1 CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

1.1.1 Pathophysiology of CLL

Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in the western hemisphere that represents 30% of all the adult leukemias. CLL is marked by progressive accumulation of CD5+ mature B cells in blood, bone marrow and secondary lymphoid organs. CLL is primarily a disease affecting the elderly population, with a median age of diagnosis around 67 years. In two-thirds of cases, CLL pathogenesis follows an indolent clinical course whereas in one-third of the cases the disease follows an aggressive course (1). CLL in some cases is preceded by a distinct premalignant clinical stage characterized by a monoclonal expansion of mature B cells, known as Monoclonal B lymphocytosis (MBL) (1). Notably, CLL cells are known to have moderate proliferation rates while having severely impaired apoptosis (1). Moreover, the survival and proliferation of CLL cells critically depends on diverse signaling cues emanating from their unique tissue microenvironment known as proliferation centers (2). The proliferation centers in CLL patients are located in Bone Marrow and Lymph nodes where they are comprised of distinct cellular and molecular components (2). The cellular components promoting the proliferation and survival of CLL cells include the monocytic lineage derived Nurse like cells, Mesenchymal stem cells, T cells as well as Macrophages. The molecular components consists of various cytokines and growth factors like IL-10, BAFF, IL-6, IFN- γ etc (2). More recently, studies have described striking, stereotypy in the usage of B cell receptor (BCR) among CLL clones from human patients (3). These studies suggest the involvement of a putative antigen that may contribute to the survival and proliferation of CLL cells. In this regard, several different self-antigens and antigens derived from pathogenic agents have been linked to the pathogenesis of CLL (4, 5). However, the identity and source of these coveted antigens remain an active area of research in the field. Together, all these

factors constitutes the specialized microenvironment involved in the complex pathogenesis of CLL.

CLL is a clinically heterogeneous disease that can be stratified into different subgroups that are associated with varied clinical outcomes (1). CLL cells carry different cytogenetic abnormalities that are shown to impact the survival of CLL patients. The 13q14 chromosomal deletion is one of the most common chromosomal alteration and is associated with deregulation of miRNA15a/16-1 and better clinical outcomes (1, 6). The 17p deletion and 11q deletions are linked to disruption of p53 and ATM functions respectively and are associated with adverse clinical outcomes (1, 6). Trisomy 12 is a chromosomal anomaly associated with intermediate prognosis, however the underlying molecular pathogenesis remains ambiguous (1, 6). CLL can be further classified into two distinct subgroups, based on the Immunoglobulin Variable Heavy Chain (IgVH) mutational status of the transformed cells (1, 6). The IgVH mutational status in B cells is reflective of their prior antigen experience. The CLL subgroup with unmutated IgVH represents cells with no prior antigen experience (unmutated CLL). On the other hand, CLL subgroup with mutated IgVH (mutated CLL) represent cells that have undergone Germinal Center reaction and have probably had prior exposure to antigens. Importantly, CLL patients with unmutated IgVH have adverse clinical outcomes compared to CLL patients with mutated IgVH. Nevertheless, the molecular events that drive CLL pathogenesis in both of these subgroups are largely unknown.

1.1.2 Cell of origin for CLL

The cell of origin for human CLL remains unclear. Several different studies have implicated diverse B cell subsets, as the cell of origin for human CLL (7). However, none of these studies so far have provided clear consensus in defining the cell of origin for human CLL. On the other hand, in multiple murine models of CLL, a specialized B cell subset known as B1 cells are presumed as the

precursors of CLL (7). B1 cells originate in the first wave of fetal liver hematopoiesis and represents the dominant mature B cell subset in neonatal mice (8). B1 cells arise from specific B1 progenitors in bone marrow and fetal liver and they are also endowed with a unique ability to undergo self-renewal to maintain their numbers (8). In adult mice, B1 cells primarily localizes to spleen, peritoneal and pleural cavities and represents only a small fraction of mature B cell population (1-5%) (8). B1 cells express a highly specialized B cell receptor repertoire that recognizes self-antigens and particulate pathogens (8). Eventually, B1 cells follow their functional fate to differentiate into short-lived plasma cells that secrete antibodies.

Until recently, the B1 cell counterparts in humans were not identified. Intriguingly, a recent study described B1 cell like B cell subsets in the peripheral blood of humans (9). Furthermore, this B1 cell like subset in humans is particularly enriched in the umbilical cord blood, consistent with their B1 cell like ontogeny. Future studies should be aimed towards defining the role of this unique B1 cell like subset, as the cell of origin for human CLL. In our studies here, we used distinct genetic models to elucidate the molecular regulation of B1 cell development and the mechanisms that lead to their transformation to CLL.

1.1.3 Mouse Models of CLL

Mouse models of CLL have contributed immensely to our current understanding of the pathogenesis of CLL. Mouse models are unique in providing a system to study CLL cells and their precursors and to follow the complete disease course which is otherwise not feasible in human CLL patients. Several different mouse models have provided valuable insights into the molecular pathogenesis of CLL. These include the well-studied New Zealand Black mouse, *Dleu2*/miR15a/16-1 deletion mouse and the $\epsilon\mu$ -TCL1 mouse (10-12). All these models share several features that are critical to the pathogenesis of human CLL as well.

The New Zealand Black (NZB) mouse strain represents a well-described naturally occurring, late-onset model of CLL (11, 13). The NZB mice develop CLL at 30-40% penetrance by the age of 12 months (11, 13). Moreover, the molecular pathogenesis of CLL in NZB mice has been shown to be associated with Single Nucleotide Polymorphisms (SNPs) that affects the processing and functional maturation of miRNAs 15a/16-1 (11). Interestingly, these miRNAs are also implicated in the pathogenesis of human CLL. The deletion of 13q14 locus, is one of the most common chromosomal abnormality present in human CLL patients (10). The 13q14 chromosomal locus in humans span the Dleu2 gene locus that also harbors miRNA15a/16-1 in the intragenic region (10). The importance of this chromosomal locus is further highlighted by the fact that the deletion of 14q13 locus in mouse which represents the region syntenic to human 13q14 leads to CLL development. The mice with 14q13 locus deletion containing the Dleu2 gene and miRNA 15a/16-1 develops CLL at 40% penetrance by the age of 18 months (10). Together, these mouse models have provided evidence for a critical role for 13q14 chromosomal locus in the molecular pathogenesis of CLL. In our studies here we used the NZB mouse as a model to study the pathogenesis of CLL.

The E μ -TCL1 is the most widely-studied mouse model of CLL. The E μ -TCL1 mouse carries a T cell lymphoma 1 (TCL1) oncogene under the control of μ Immunoglobulin (Ig) heavy chain enhancer (12). Even though, the E μ -TCL1 mouse has been extensively used to study CLL, the role of Tcl1 oncogene in human CLL remains an active area of investigation. Moreover, these mice develop CLL at near complete penetrance and have been used to extensively study the molecular pathogenesis and efficacy of therapeutic agents in CLL. The CLL clones arising from these mice display considerable BCR stereotypy, with the majority of clones expressing the Vh11 Immunoglobulin heavy chain family (12, 14). Interestingly, the Vh11 Immunoglobulin heavy chain gene family is predominantly expressed by B1 cells in mice (15). In our studies here we used a

genetically engineered BCR knock-in mouse expressing the Vh11 Ig heavy chain as a model system to study the development of CLL precursors (B1 cells) and their transformation to CLL.

1.1.4 Molecular Pathogenesis of CLL

The molecular pathogenesis of CLL has not been fully elucidated. With the advent of Next Generation Sequencing, there is plethora of high resolution genetic information available that implicates multiple pathways in the pathogenesis of CLL. Additionally, several studies have also implicated multiple other factors in the molecular etiology of CLL. These include genetic predisposition, autoantigen stimulation, microRNAs (miRNAs), and cytogenetic abnormalities (6, 16). However, only a few of these abnormalities have been conclusively linked to the pathogenesis of CLL. Therefore, supplementing these findings with additional experimental evidences is quintessential to fully understand the roles of these factors in CLL biology. In our studies here we aimed to identify the molecular components that are critical in the etiology of CLL.

1.2 Interferon Regulatory Factor 4 and CLL

Interferon Regulatory Factor 4 (IRF4) belongs to Interferon Regulatory Factor (IRF) superfamily of transcription factors that plays critical roles at multiple stages of B cell development. Studies spanning over more than the past decade have linked IRF4 to pathogenesis of CLL. An initial study described that low levels of IRF4 in CLL cells are correlated with poor disease prognosis (17). Additionally, a recent study also identified mutations in the DNA binding domain of IRF4 in a small subset of CLL patients (~1.5%) (18). However, the functional consequences of these mutations to IRF4 function in B cells remain ambiguous. Most intriguingly, a Genome Wide Association Study (GWAS) in CLL patients identified IRF4 as a major susceptible gene for CLL development (19). This study identified strong associations between the Single Nucleotide Polymorphisms (SNPs) in IRF4

gene locus and the risk of developing CLL (19). Moreover, functional analysis revealed that the risk alleles carrying these SNPs mediate the downregulation of IRF4 mRNA in Epstein-Barr Virus transformed B cells (19). Importantly, the SNPs in IRF4 gene locus were found to be highly prevalent, accounting for 86% of all the CLL cases (19, 20). The high incidence of occurrence of these SNPs in CLL patients are suggestive of an important function of IRF4 in CLL biology. Together, these studies provide evidence for a link between low levels of IRF4 and CLL development. Yet the precise role of IRF4 in CLL development was largely unknown. In our studies here, we used genetic approaches to examine the role of IRF4 in the pathogenesis of CLL.

1.3 IRF4 and its role in B cell development

Interferon regulatory factor 4 (IRF4) functions as a critical transcriptional regulator for development and function of several immune cell subsets. IRF4 is most homologous to an IRF family member, IRF8. Structurally, IRF4 contains a tryptophan pentad DNA binding domain (DBD) and an interferon association domain (IAD) through which it can homo- or hetero-dimerize with other members of the IRF family (21, 22). To perform its transcriptional regulatory functions, IRF4 can form homo- or hetero-dimers with itself or with other members of the IRF family. The IRF4 homodimers bind DNA with low affinity at canonical Interferon-Stimulated Response Elements (ISRE) represented as GAAANNGAAA (21, 22). Besides its interaction with other IRFs, IRF4 can also form heterodimers with members of Ets family or AP-1 family of transcription factors (21, 22). The heterodimers formed between IRF4 and Ets members, PU.1 and Spi-B bind DNA at Ets Interferon Composite Elements (EICE) represented as GGAANN(N)GAAA. The EICE motifs were initially identified in immunoglobulin (Ig) light chain 3' κ enhancer and λ enhancer regions mediating Ig light chain locus activation (23, 24). The IRF4-Ets hetero-dimers bind to DNA at EICE

motifs with much greater affinity than ISRE motifs (25). More recently, IRF4 has been shown to co-bind DNA with AP-1 family members on AP-1-IRF Composite Elements (AICE) represented as GAAATGAGTCA or GAAANNNTGAGTCA in a variety of immune cell subsets (24-26). The formation of complexes between IRF4 with either Ets or Ap-1 transcription factors depends on the cell type and cellular context. For example, the Ap-1-IRF complexes are predominantly known to regulate cellular functions in T cells and dendritic cells while Ets/IRF complexes are critical for B cell development and functions. The cooperative DNA binding of IRF4 with members of IRF, Ets and AP-1 families represents an evolutionarily conserved mechanism to integrate diverse signaling inputs during immune system development and function (26).

IRF4 is an important regulator of generation, differentiation and functions of several immune cell subsets. IRF4 play key roles in generation and functions of T follicular helper cells (Tfh), Th1 cells, Th2 cells, Th9 cells, T regulatory cells, CD8+ T cells, Th17 cells, macrophages and dendritic cells (27-41). In B cells specifically, IRF4 is expressed at multiple stages to control important developmental decisions (21). At early stages of B cell development IRF4 functions redundantly with IRF8 to coordinate pre-B cell differentiation (42). On the other hand, at later stages of B cell development, IRF4 and IRF8 have been shown to function non-redundantly to regulate follicular versus marginal zone cell fate decisions, germinal center reaction (GC), class switch recombination (CSR) and plasma cell differentiation (21, 25, 43-46).

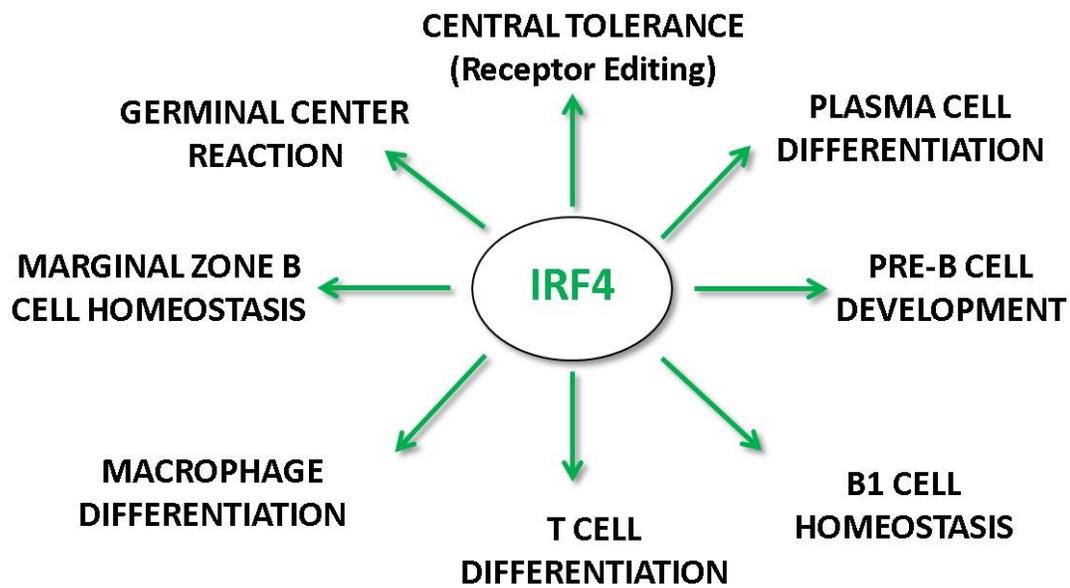


Figure 1 Schematic showing the various developmental aspects of Immune Cell subsets that are regulated by IRF4.

Consistent with a critical involvement of IRF4 in B cell development, deregulated expression of IRF4 is associated with pathogenesis of several B cell malignancies and diseases. IRF4 is known to play diverse yet distinctive roles in B cell malignancies. In early B cell derived acute lymphoblastic leukemia (B-ALL), IRF4 functions as a tumor suppressor (47, 48). Moreover, in multiple myeloma (MM) that originates from plasma cells, it acts as a survival factor (49). Furthermore, studies have linked low levels of IRF4 to CLL development. However, whether and how low levels of IRF4 contributes to CLL development remained unknown. In our studies here we studied the role of IRF4 in the maintenance of B1 cell homeostasis and the development of CLL.

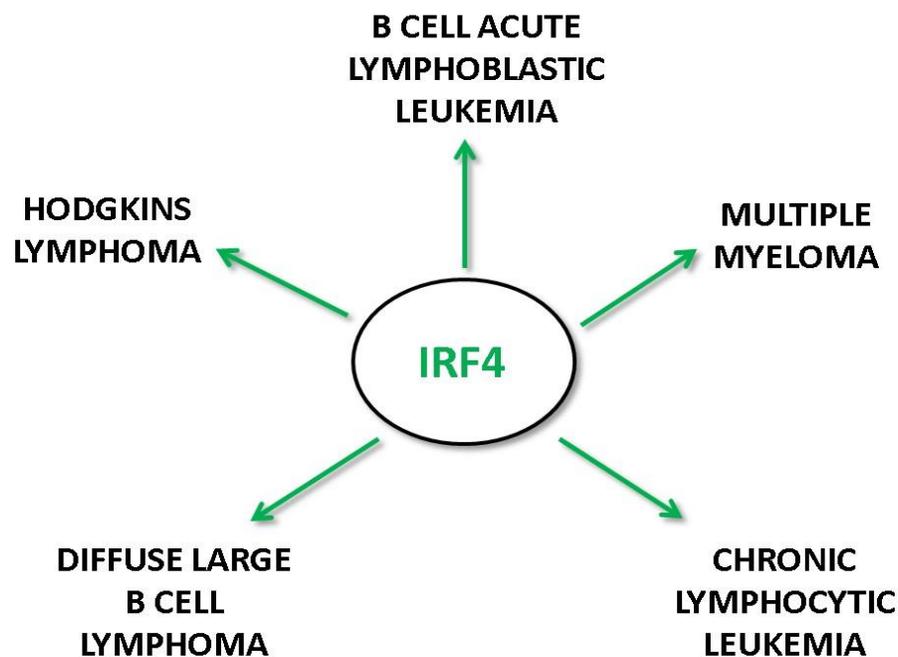


Figure 2 A schematic showing the various B cell malignancies in which IRF4 is implicated.

1.4 Hypothesis and Approaches I

Our studies here were aimed towards elucidating the role of IRF4 in the development of CLL. We hypothesized that **low levels of IRF4 promotes the initiation and progression of CLL and are causally related to the development of CLL**. To test our hypothesis we used distinct genetic approaches. First, we studied the role of low levels of IRF4 in the development of CLL in NZB mice. These studies allowed us to examine, how IRF4 collaborates with the preexisting genetic defects in the NZB mice to impact CLL development. Second, we used a Vh11 knock-in mouse that has been previously described as model system to study B1 cells (CLL precursors). These studies allowed us to investigate the role of IRF4 in the development of B1 cells and CLL initiation.

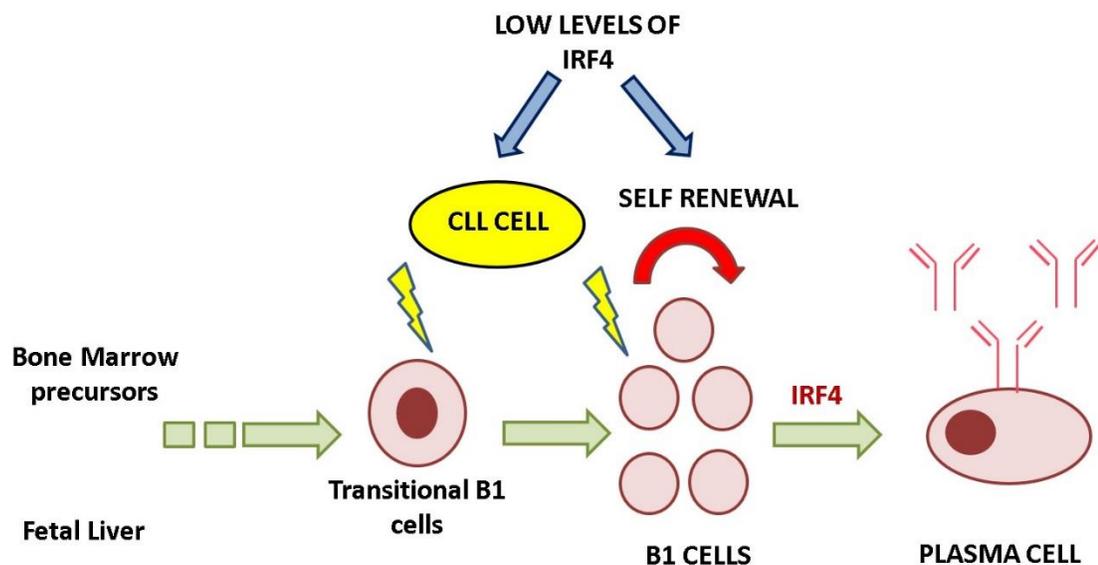


Figure 3 Hypothesis. A schematic representation of the hypothesis that was tested in our studies. B1 cells originate from fetal liver or bone marrow precursors and have a unique property to self-renew themselves. Mature B1 cells eventually differentiate to plasma cells, a process that absolutely requires IRF4. Low levels of IRF4 may affect the proliferation and survival of CLL cells and their precursors contributing to the initiation and progression of CLL.

1.5 Notch signaling and CLL development

Recent Whole Genome Sequencing (WGS) studies have provided valuable insights into the molecular pathways deregulated during the pathogenesis of CLL (50-53). Intriguingly, multiple WGS studies have identified mutational activation of Notch signaling pathway as one of the most recurrent molecular events in human CLL (51, 53). Moreover, the CLL patients carrying mutations in Notch signaling pathway have poorer clinical outcomes and an increased tendency towards Richter transformation to Diffuse Large B cell Lymphoma (DLBCL) (50).

Notch signaling is an evolutionarily conserved pathway that regulates a myriad of cellular processes (54). Notch family of proteins comprises of four different Notch receptors (Notch1 through 4) in mammalian cellular systems. Notch signaling is activated upon engagement of Notch receptor on a signal receiving cell with its ligand on a signal sending cell (54). Following the ligand binding Notch receptor undergoes multiple proteolytic cleavages leading to translocation of intracellular domain of Notch to the nucleus (54). In the nucleus Notch intracellular domain functions as a transcriptional regulator to activate gene transcription of its target genes (54). In CLL patients, Notch signaling pathway is activated due to mutations primarily affecting the stability of Notch1 protein (51, 53). Notch mutations in CLL patients causes frameshift deletions leading to generation of protein without the PEST domain (51, 53). The PEST domain in Notch proteins are functionally important for ubiquitination and degradation of Notch proteins and hence for limiting the cellular responses to Notch signaling (54). Other than the mutational activation, studies have also reported constitutively high expression of Notch1 and Notch2 leading to activation of Notch signaling in human CLL cells (55). Moreover, *In vitro* studies relying on co-culture of CLL cells with stromal cells have also provided evidence for a role of Notch signaling in promoting the survival and chemo-resistance of CLL cells (56, 57). Although, these studies have linked aberrant Notch signaling to the pathogenesis of CLL *in vitro*, whether Notch signaling is

critical for CLL development *in vivo* remains unknown. Furthermore, the molecular mechanism leading to the deregulation of Notch signaling in CLL is still ambiguous.

A recent study described expansion of a specialized mature B cell subset known as Marginal Zone B cells (MZ B cells) in IRF4 deficient mice (45). Intriguingly, IRF4 deficient mature B cells were found to display higher levels of Notch2 receptor and associated Notch signaling leading to expansion of MZ B cells (45). Although, these studies identify IRF4 as a negative regulator of Notch signaling in mature B cells, the precise mechanism of modulation of Notch signaling by IRF4 remains unclear. In our studies here, we tested the significance of IRF4-Notch axis in the development of CLL.

1.6 Hypothesis and Approaches II

These studies were aimed towards defining the importance of Notch signaling in the development of CLL. Furthermore, these studies were also directed towards establishing the role of IRF4 in inhibiting the responses of CLL cells and their precursors to Notch signaling. We hypothesized that **IRF4 functions as an attenuator of Notch signaling to suppress CLL development**. To test our hypothesis we used genetic approaches to study the significance of Notch signaling during CLL development in the presence of low levels of IRF4. Additionally, we used Next Generation Sequencing approaches to identify the molecular mechanisms by which IRF4 inhibits Notch signaling in CLL cells and their precursors.

1.7 Significance

The high prevalence of IRF4 risk alleles in CLL patients point towards a central role for IRF4 in the pathogenesis of CLL. However, the precise role of IRF4 in the development of CLL was unclear. Our studies here used distinct genetic models to elucidate the role of IRF4 in the development of CLL.

Notch signaling has been identified as one of the most recurrently mutated pathways in CLL. Furthermore, studies have identified constitutive activation of Notch signaling pathway as a hallmark of CLL. These studies provide evidence for an important role of Notch signaling in the pathogenesis of CLL. However, the significance of Notch signaling in the development of CLL *in vivo* is still unknown. Moreover, the molecular mechanisms underlying the deregulation of Notch signaling in CLL remains unclear. In our studies here we explored the role of IRF4 as an attenuator of Notch signaling during the development of CLL.

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CHAPTER 2

Accelerated development of chronic lymphocytic leukemia in New Zealand Black mice expressing low level of interferon regulatory factor 4.

The material presented in this chapter has been previously published: Shibin Ma^{1#}, Vipul Shukla^{1#}, Fang Leilei¹, Karen A Gould¹, Shantaram S. Joshi¹ and Runqing Lu¹ “Accelerated development of CLL in NZB mice expressing low levels of IRF4.” *Journal of Biological Chemistry (JBC)* **2013 Sep 13;288(37):26430-40. doi:10.1074/jbc.M113.475913 # co-first authors**

2.1 INTRODUCTION

Chronic lymphocytic leukemia (CLL) 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 (miRNAs), and cytogenetic abnormalities (2). However, the molecular basis of CLL pathogenesis has not been fully elucidated, largely because few genetic abnormalities have been conclusively linked to the development of CLL. Among the common genetic defects in CLL, only 13q14 deletion has been conclusively linked to the pathogenesis of CLL. 13q14 deletion is the most common genetic abnormality in CLL and is found in 50 to 60% of cases (2). As such, 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 initially thought 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 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). Once 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 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.

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 cells 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 downregulation of IRF4 in CLL (20). Low levels of IRF4 in CLL cells were also found to be correlated with poor prognosis in CLL patients (21). Finally, mutations in the DNA binding domain of 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 if 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 to wild type NZB mice (NZB IRF4^{+/+}), CLL development was dramatically accelerated 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.

2.2 MATERIALS AND METHODS

Mice. IRF4 heterozygous mutant mice (IRF4^{+/-}) have been previously described (23). The Rag2 and common gamma chain double deficient mice (Rag2^{-/-}γ^{-/-}) mice were obtained from Taconic. NZB mice were obtained from Jackson lab. 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 University of Nebraska Medical Center. The mice aged from 5 to 30 weeks were used for this study.

FACS analysis and cell sorting. Cells were isolated from NZB IRF4^{+/+} and NZB IRF4^{+/-} mice, pre-incubated with either 2% rat serum or Fc-Block (2.4G2), and stained with 15 nanograms of respective antibodies per million cells that were either biotinylated or directly fluorochrome-conjugated. All the antibodies were purchased from BD Pharmingen. FACS analysis was performed with a FACS Calibur flow cytometer.

Adoptive transfer of B1 cells. Cells were isolated from PCs of NZB IRF4^{+/+} and NZB IRF4^{+/-} mice and were incubated in tissue culture dishes containing RPMI-1640 media for 4 hours to remove adherent macrophages. The cells in suspension were collected and stained with CFSE dye and were injected into 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 Pharmingen following manufacturer's instructions. 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 i.p. with 6 mg/ml BrdU (Sigma-Aldrich) and 12 hours 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 detected with a BrdU flow kit (Pharmingen). 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 terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. The TUNEL assay was conducted as previously described (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 Pharmingen). The 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 Pharmingen.

Assay to measure phospho-Akt. MEC-1 cells were fixed in 2% paraformaldehyde for 10 minutes and permeabilized in 100% methanol for 30 minutes. The permeabilized cells were incubated with anti-phospho-Akt^{S473} antibody (Alexa Fluor® 488 Conjugate, Cell signaling) for 1 hour 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 Taqman MicroRNA Reverse Transcription kit and Taqman RT primers (ABI). For miRNA quantification, TaqMan miRNA assays (ABI) were used according to the manufacturer's protocol. 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 S17 stromal layer in media 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 x10⁶ CLL cells and 20µg of plasmid were used for each transfection. The transfection was carried out in a Nucleofector (Lonza) with Solutioin V using program G-016. The transfected cells were analyzed 48 hours later. For transfection of human MEC-1 cells, 2 x 10⁶ cells and 20 µg of plasmid were used for each transfection. The condition for MEC-1 transfection is Solution V and program X-001. 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 150aa).

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 Probes) for 30 minutes at 37 °C in RPMI 1640 media containing 3 % FBS. The Calcium influx of loaded cells was analyzed with a LSR II flow cytometer. The baseline emission of fluorescence ratio (405:525 nm) of CLL or B1 cells was collected for 1 min. Then anti-µ antibody (Jacksonimmuno Res) at 5 µg/ml was added and fluorescence ratio was recorded for another 4 minutes. 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 (Jacksonimmuno) at 37°C for 5 minutes. The cells were lysed and proteins were separated by SDS-PAGE gel. The membranes were incubated with indicated antibodies and the signals were revealed with ECL detection

system (Pierce). The antibodies against Akt, phospho-Akt^{S473}, Erk, phospho-Erk, Tcl-1, Bcl-2, Bcl-xl and Mcl-1 were obtained from Cell Signaling. The antibody against IRF4 was purchased from Santa Cruz Biotech.

2.3 RESULTS

2.3.1 Precocious CLL development in NZB mice expressing low levels of IRF4.

We have been using 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 C57B6 background onto NZB background for at least 7 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. A total of ten mice from each group were used. The appearance and percentage of a monoclonal B220^{low}CD5⁺ in the peripheral blood mononuclear cells (PBMC) were used for the initial CLL diagnosis. The diagnostic criteria for CLL in NZB mice were a monoclonal population of B220^{low}CD5⁺ cells constituting over 20% of PBMC. If the percentage of the B220^{low}CD5⁺ clone was under 20% of the PBMC, a diagnosis of monoclonal B cell lymphocytosis (MBL).

The latency to onset of CLL in NZB IRF4^{+/-} mice is typically around 12 months. Interestingly, two out of ten NZB IRF4^{+/-} mice developed CLL after just three months (Fig. 1A). After five months, eight out of ten NZB IRF4^{+/-} mice developed CLL while the remaining two developed MBL. The NZB IRF4^{+/-} mice with MBL all progressed into CLL within 10 month. 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 month (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 B220^{low}CD5⁺ cells. However, presence of significant amount of B220^{low}CD5⁺ CLL cells was also detected in the blood, bone marrow and spleen of NZB IRF4^{+/-} mice. In NZB IRF4^{+/-} mice, 80% of splenocytes were B220^{low}CD5⁺ CLL cells (Fig.1B). The infiltrations of CLL cells in spleens of NZB IRF4^{+/-} mice caused massive splenomegaly where an average accumulation of $3.6 \pm 1.4 \times 10^8$ CLL cells was observed. Besides 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 dramatically accelerated in the NZB IRF4^{+/-} mice.

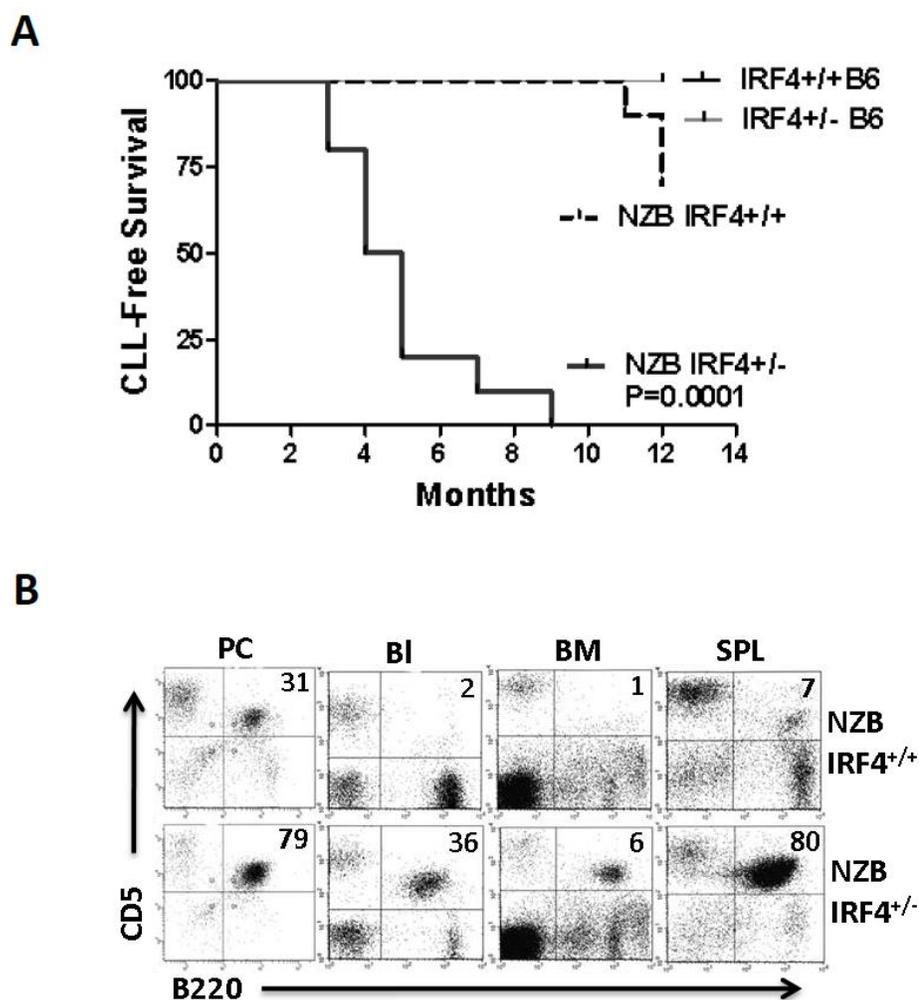


Figure 1 Precocious CLL development in NZB mice expressing reduced level of IRF4. A) Kaplan-Meier CLL-free survival curve of IRF4^{+/+}B6, IRF4^{+/-}B6, NZB IRF4^{+/+} and NZB IRF4^{+/-} mice. 10 mice from each genotype were used for the analysis. P value for pair-wise comparison (log rank test) between NZB IRF4^{+/+} and NZB IRF4^{+/-} is 0.0001. Graphpad PRISM 5.03 was used to plot the survival curve and to calculate p value. B) Cells were isolated from peritoneal cavity (PC), blood (BL), bone marrow (BM), and spleen (SPL) of five-month old NZB IRF4^{+/+} and NZB IRF4^{+/-} mice. The isolated cells were stained with antibodies against CD5 and B220 and analyzed by FACS. The numbers are frequency of CLL cells.

2.3.2 Phenotypical and histological characterization of NZB IRF4^{+/-} CLL cells.

To further characterize CLL cells in the 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 which is normally expressed at higher levels on follicular B cells (FO B) but at low levels on B1 cells. CD21 and CD23 are markers for FO 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 FO B cells. Our result shows that NZB IRF4^{+/-} CLL cells were B220^{low}IgM^{hi} IgD^{low}CD21⁻CD23⁻CD43⁺, a surface phenotype that resembles B1 cells from which they were derived (Fig.2A). We also performed histological analysis of spleens of NZB IRF4^{+/-} mice. Histological examination of spleen of NZB IRF4^{+/-} mice revealed a grossly distorted white pulp and red pulp, with larger, irregular lymphoid follicles (Fig. 2B). Under high magnification (40x), the splenic lymphocytes in NZB IRF4^{+/-} mice (predominantly CLL cells) were slightly bigger than those in the NZB IRF4^{+/+} mice.

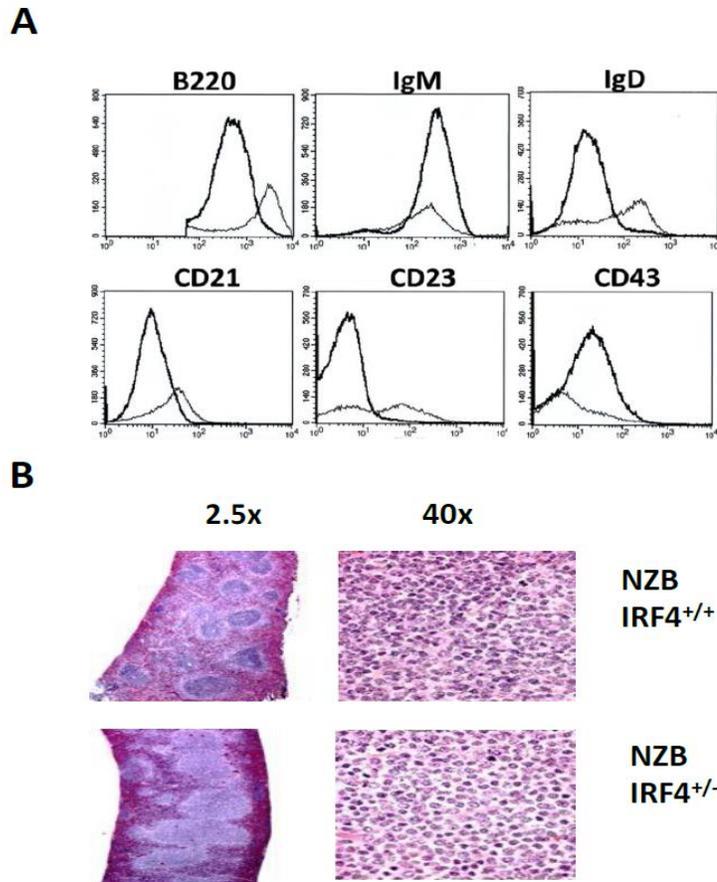


Figure 2 Phenotypal and histological characterizations of NZB IRF4^{+/-} CLL cells. A) Splenocytes from NZB IRF4^{+/+} and NZB IRF4^{+/-} mice were stained with antibodies against B220, CD21, CD23, IgM, IgD and CD43. The stained cells were analyzed by FACS. The data were presented as histogram under a B cell gate. Dark line: NZB IRF4^{+/-} CLL cells; light line: NZB IRF4^{+/+} B cells. B) Spleens were isolated from five-month old NZB IRF4^{+/-} and NZB IRF4^{+/+} mice. The tissues were fixed, sectioned, stained with hematoxylin and eosin (H&E). The stained tissues were examined under a ZEISS Axioplan 2 Microscope and analyzed with AxioVision software. The spleen sections are shown at 2.5x and 40x.

2.3.3 NZB IRF4^{+/-} CLL cells expand predominantly in 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). Since 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 node and spleen 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 node. These results indicate that blood, bone marrow and lymph node 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 spleen. This finding is consistent with result of BrdU pulse-labeling analysis which shows that 9% of CLL cells in spleen were BrdU positive while percentages of BrdU+ CLL cells in BL, BM and LN 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 the 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 spleen and are resistant to apoptosis.

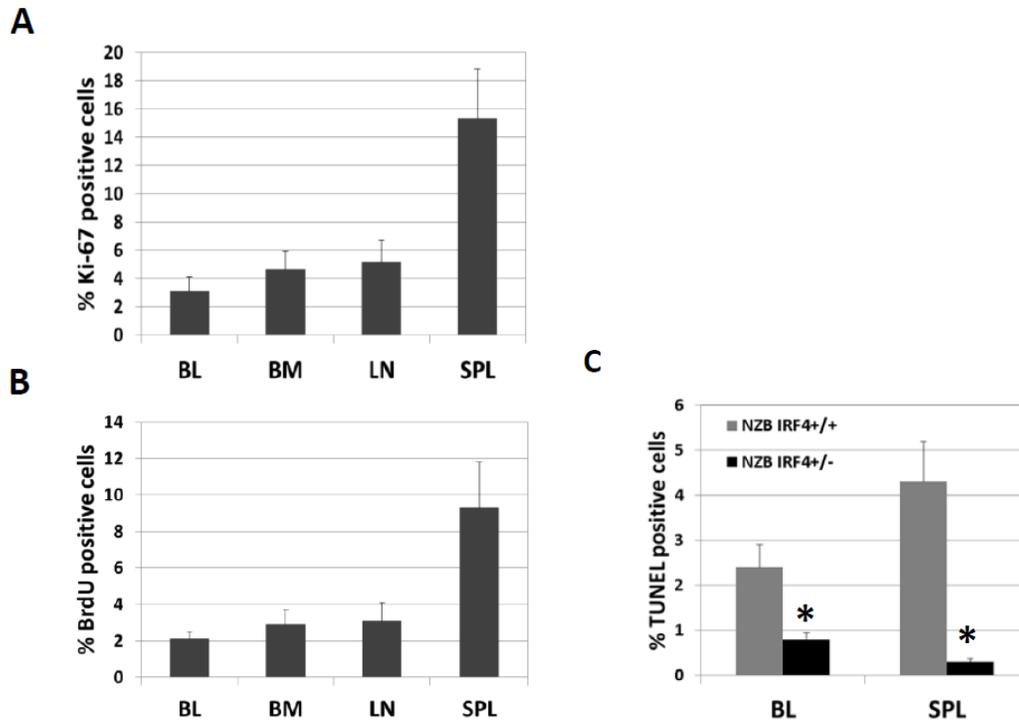


Figure 3 NZB IRF4^{+/-} CLL cells expand predominantly in spleen and are resistant to apoptosis. A) CLL cells were isolated from BL, BM, lymph node (LN) and SPL of five-month old NZB IRF4^{+/-} mice and stained with antibodies against CD5, IgM and Ki67. The Ki67 positive cells were revealed by FACS. The data were averages and standard deviations of three independent experiments. B) NZB IRF4^{+/-} mice were pulse-labeled with BrdU for 12h. After staining, BrdU positive CLL cells in BL, BM, LN and SPL were revealed by FACS. C) Cells were isolated from BL and SPL of five-month old NZB IRF4^{+/-} and NZB IRF4^{+/+} mice and stained with antibodies against CD5 and IgM. The apoptotic cells were revealed by TUNEL assay. The data were averages and standard deviations of three independent experiments. * $p < 0.01$.

2.3.4 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 splenic B1 cells of NZB IRF4^{+/+} mice were used as controls. The cells were stained with Indo-1 Am, stimulated with anti-IgM antibodies and analyzed by LSR flow cytometer. As shown in Fig. 4A, BCR signaling-induced Ca²⁺ influx was more potent in the NZB IRF4^{+/-} CLL cells than in the IRF4^{+/+} NZB splenic B1 cells, indicating that NZB IRF4^{+/-} CLL cells are hyperresponsive to BCR stimulation. We further measured the activation of Akt and Erk, two important downstream mediators of BCR signaling, in isolated splenic CLL cells and control cells. Engagement of BCR led to enhanced phosphorylation of Akt and Erk. Anti-IgM cross-linking induced more robust phosphorylation of both Akt and Erk in NZB IRF4^{+/-} CLL cells than in IRF4^{+/+} NZB splenic B cells (Fig 4B). Collectively, these results indicate that NZB IRF4^{+/-} CLL cells are hyperresponsive to BCR stimulation. We wanted to further determine whether BCR signaling can be regulated by expression level of IRF4. To this end, we raised the expression level of IRF4 in the NZB IRF4^{+/-} CLL cells via transient transfection. MigR1 is a retroviral vector that co-expresses GFP. MigIRF4 and MigR1 control vector were transduced into NZB IRF4^{+/-} CLL cells via a Nucleofector. The transfection efficiencies were about 10% in the MigR1 transduced cells but only 3% in the MigIRF4 transduced cells. Two days after transfection, the cells were stained with Indo-1 Am and stimulated with anti-IgM antibodies. The Ca²⁺ influx in GFP⁺ cells was examined (Fig. 4C). Our result shows that the Ca²⁺ influx triggered by BCR signaling was comparable between MigR1 and MigIRF4 transduced cells, indicating that transient increase of IRF4 expression level cannot attenuate BCR signaling in the NZB IRF4^{+/-} CLL cells.

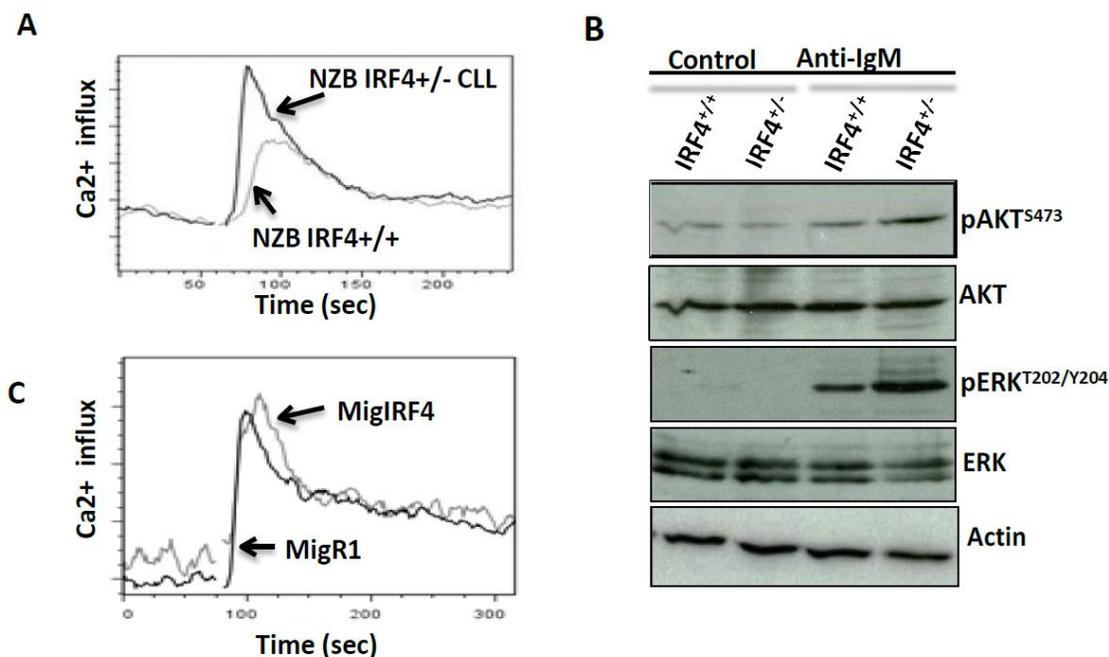
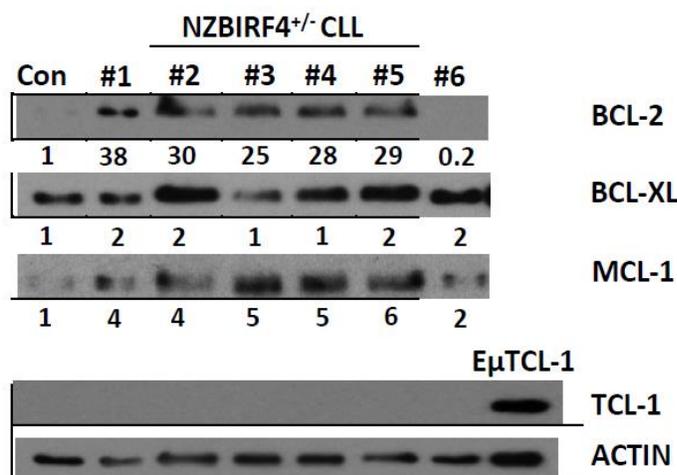


Figure 4 NZB IRF4^{+/-} CLL cells are hyperresponsive to BCR stimulation. A) Isolated splenocytes from NZB IRF4^{+/-} and NZB IRF4^{+/+} mice were stained with antibodies against B220 and CD5. The stained cells were incubated with Indo-1 Am for 20 minutes at 37°C. The Calcium influx induced by 5µg/ml anti-IgM antibodies were recorded by a LSRII flow cytometer. The Calcium influx by CLL cells and B1 cells were overlaid with Flowjo software. Dark line: NZB IRF4^{+/-} CLL cells; light line: NZB IRF4^{+/+} 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 10µg/ml for 5 minutes. Splenic B cells isolated from NZB IRF4^{+/+} mice were analyzed as control. Western blot analysis was performed to detect phospho-Erk and Akt. C) CLL cells were isolated from spleen of NZB IRF4^{+/-} mice and plated on top of S17 stromal cells in RPMI-1640 media 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 5µg/ml anti-IgM. Ca²⁺ influx in GFP+ cells was examined. MigR1 and MigIRF4 transfected cells are indicated by arrows.

2.3.5 Molecular characterization of NZB IRF4^{+/-} CLL cells.

To further characterize NZB IRF4^{+/-} 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^{+/-} mice and expression of Bcl-2 family members Bcl-2, Bcl-xl and Mcl-1 was examined by Western blot. Splenic B cells from NZB IRF4^{+/+} mice were used as control. Similar to human CLL cells, expression of Bcl-2 and Mcl-1 was significantly elevated in all six CLL samples (Fig.5A). In contrast, expression of Bcl-xl was only moderately increased in some but not all samples. 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 Tcl1 oncogene in B cells (E μ Tcl1) develop late onset, aggressive CLL (29). It has been shown that TCL1 oncogene can promote survival of CLL cells by directly interacting with Akt (30, 31). However, except for CLL cells isolated from E μ Tcl1 transgenic mice, expression of Tcl1 cannot be detected in samples derived from NZB IRF4^{+/-} CLL cells, indicating that expression of Tcl1 is very low in those samples. Additionally, Total RNA was isolated from NZB IRF4^{+/-} CLL cells and expression of miR15a and 16-1 were measured by real time PCR. RNA isolated from splenic B cells of NZB IRF4^{+/+} mice was examined and used as control. Among the four CLL samples examined, expression levels of miR15a/16-1 were comparable with that of control (Fig.5B). In summary, our results show that expression of Bcl-2 and Mcl-1 are significantly elevated in the NZB IRF4^{+/-} CLL cells, however, expression of Tcl1 and miR15a/16-1 are not deregulated in these cells.

A



B

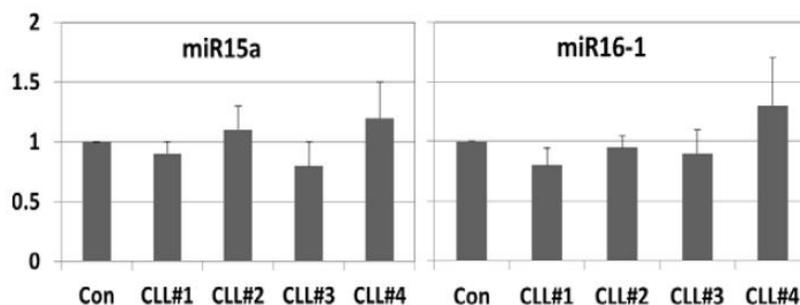


Figure 5 Molecular characterization of NZB IRF4^{+/-} CLL cells. A) Splenic CLL cells were isolated from six NZB IRF4^{+/-} mice via negative selection and lysed for Western blot analysis with indicated antibodies. Splenic B cells from NZB IRF4^{+/+} mice were also isolated and analyzed as control (Con). Additionally, splenic CLL cells from EμTcl1 transgenic mice were used as 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 fold change in comparison to the control.

2.3.6 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 reduced level of IRF4 on normal B1 cell development and function. For this study, we used 5-6 weeks old NZB IRF4^{+/-} mice before they develop CLL. Compared to NZB IRF4^{+/+} mice, the number of B1 cells in NZB IRF4^{+/-} mice increased significantly in PC; the number of B1 cells were $8.2 \pm 3.8 \times 10^6$ in NZB IRF4^{+/-} mice but only $4.1 \pm 2.1 \times 10^6$ in the NZB IRF4^{+/+} mice (Fig. 6A). This is an indicative of defects in B1 cell homeostasis in the NZB IRF4^{+/-} mice. We further analyzed the survival and expansion of B1 cells in IRF4^{+/-} NZB mice. For 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 were cultivated *in vitro* for 24h. The apoptotic cells were revealed by Propidium Iodide (PI) staining. While $15 \pm 3.2\%$ NZB IRF4^{+/+} B1 cells underwent apoptosis, only $5.8 \pm 1.7\%$ NZB IRF4^{+/-} B1 cells were apoptotic, indicating that NZB IRF4^{+/-} B1 cells are more resistant to apoptosis (Fig. 6B). B1 cell homeostasis in the PC is regulated by a balance between survival and self-renewal. To measure B1 cell expansion (self-renewal), we isolated B1 cells from PCs of NZB IRF4^{+/+} and NZB IRF4^{+/-} mice. The isolated B1 cells were stained with CFSE dye and transplanted into PCs of Rag2^{-/-}γ^{-/-} deficient host mice. Ten days later, the transplanted B1 cells were isolated and analyzed by FACS. Based on the dilution of CFSE dye, NZB IRF4^{+/-} B1 cells expanded at a much faster rate in host mice than 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 lipopolysaccharide (LPS). Three days later, differentiated plasma cells (CD138⁺) were revealed by FACS. Whereas 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.

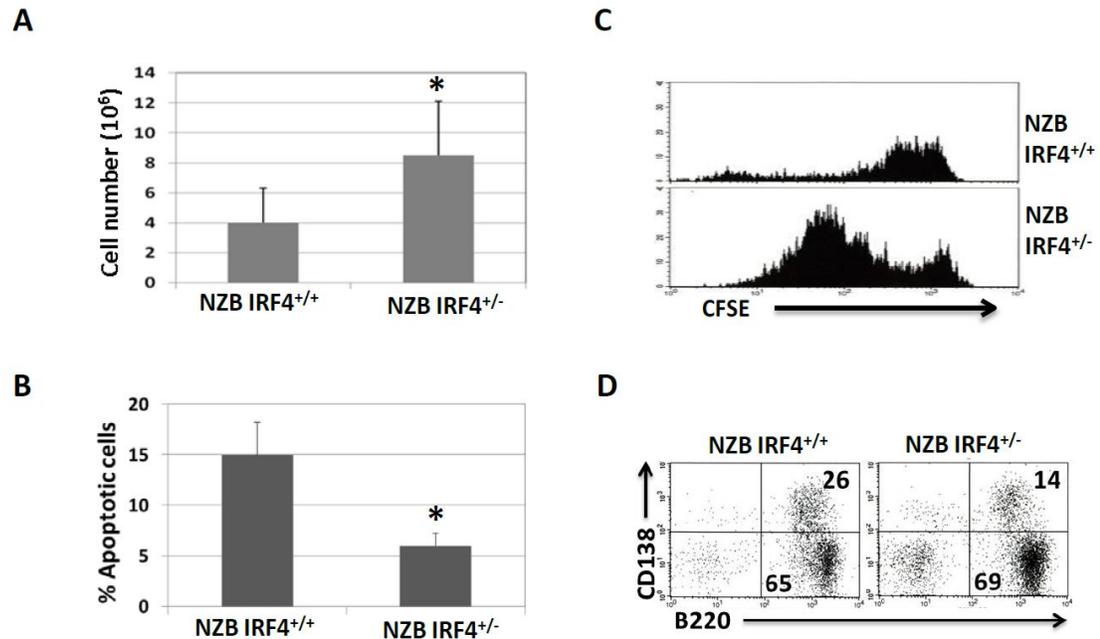


Figure 6 Defects in B1 cell survival, expansion and differentiation in the NZB IRF4^{+/-} mice. A) Cells were isolated from PCs of NZB IRF4^{+/+} and NZB IRF4^{+/-} mice and were counted. The numbers are averages and standard deviations of five mice from each group. * p<0.01. B) Isolated B1 cells were cultivated in RPMI1640 media for 24h. PI staining was used to identify the apoptotic cells. * p<0.01. C) B1 cells were isolated from PCs of NZB IRF4^{+/+} and NZB IRF4^{+/-} mice and stained with CFSE dye. The stained cells were injected into PCs of Rag2^{-/-}γ^{-/-} deficient mice at 10⁵ cells per injection. 10 days later, the transplanted cells were isolated for FACS analysis. D) Isolated B1 cells from NZB IRF4^{+/+} and NZB IRF4^{+/-} mice were cultivated in vitro in presence of LPS (5μg/ml). Three days later, the cells were stained with antibodies against CD138 and B220 and analyzed by FACS.

2.3.7 High levels of IRF4 expression inhibit survival of NZB IRF4^{+/-} CLL cells.

Expression level of IRF4 is presumably reduced by 50% in the NZB IRF4^{+/-} mice. To confirm this, we measured the expression levels of IRF4 in isolated NZB IRF4^{+/-} CLL clones. Compared to NZB IRF4^{+/+} B1 cells, the expression levels of IRF4 were reduced by 50 to 60 % in CLL clones isolated from NZB IRF4^{+/-} mice (Fig. 7A). This result confirmed that IRF4 is expressed at reduced levels in the NZB IRF4^{+/-} CLL cells. Our result shows that NZB IRF4^{+/-} CLL cells were 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 the NZB IRF4^{+/-} CLL cells via Nucleofector (described in Fig. 4C). While 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 expression level of IRF4 transcript was about three-fold higher in the 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.

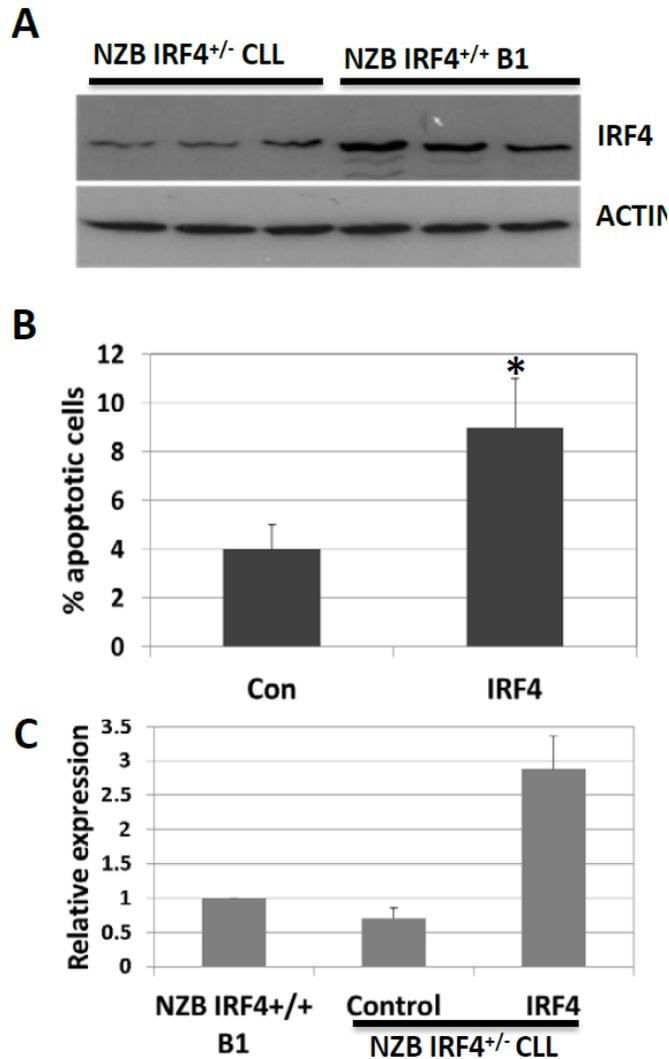


Figure 7 High levels of IRF4 inhibit the survival of NZB IRF4^{+/-} CLL cells. A) Protein lysates from three independent NZB IRF4^{+/-} CLL clones as well as three independent NZB IRF4^{+/+} B1 cells were used for Western blot analysis. The expression levels of IRF4 and β -actin control were examined with specific antibodies. B) CLL cells were isolated from spleen of NZB IRF4^{+/-} mice and plated on top of S17 stromal cells in RPMI-1640 media containing 10% FBS. MigIRF4 and MigR1 vectors were transfected into cultivated CLL cells using a Nucleofector. 48h later, the apoptotic cells in successfully transfected CLL cells (GFP+) were analyzed by detecting activated caspase3. The results were averages and standard deviations of three independent experiments. * $p < 0.01$. C) Control and IRF4 transfected cells were isolated by sorting and expression levels of total IRF4 transcript in these cells were measured by real time PCR. The values were presented as fold change in comparison to expression level of IRF4 in B1 cells isolated from NZB IRF4^{+/+} mice.

2.3.8 High levels of IRF4 expression suppress Akt activity and promote apoptosis in human CLL cells.

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 IRF4 expression level on human CLL cells. MEC-1 is the most widely used human CLL cells (32). Expression level of IRF4 is significantly lower in the MEC-1 cells than in 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 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. While only 4% of MigR1 transfected cells were Annexin-V positive, 18% of MigIRF4 transfected cell underwent apoptosis, indicating that 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 the MEC-1 cells. Unlike IRF4, high level of IRF8 did not affect the survival of MEC-1 cells. Since the target gene and GFP are translated from the same mRNA transcript through an internal ribosome entry site (IRES), 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 level of GFP whereas IRF4 and IRF4Del transduced cells expressed the lowest level (Fig. 8C).

It has been shown that PI3 kinase (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 Ser⁴⁷³ is required for its activation. A recent study has shown that Akt is constitutively phosphorylated (Ser⁴⁷³) in MEC-1 cells and inhibition of Akt phosphorylation dramatically reduces 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 (Ser⁴⁷³) in MEC-1 cells transfected with MigR1, IRF4 and IRF4Del. MEC-1 cells were also treated with PI3k inhibitor LY294002 and analyzed as control. As shown in Fig. 8D, LY294002 treatment decreased the percentage of phospho-Akt expressing cells from 83% in untreated cells to 29% in the treated cells. Additionally, LY294002 treatment also led to enhanced cell apoptosis (data not shown). While 70% phospho-Akt expressing cells were found in the 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.

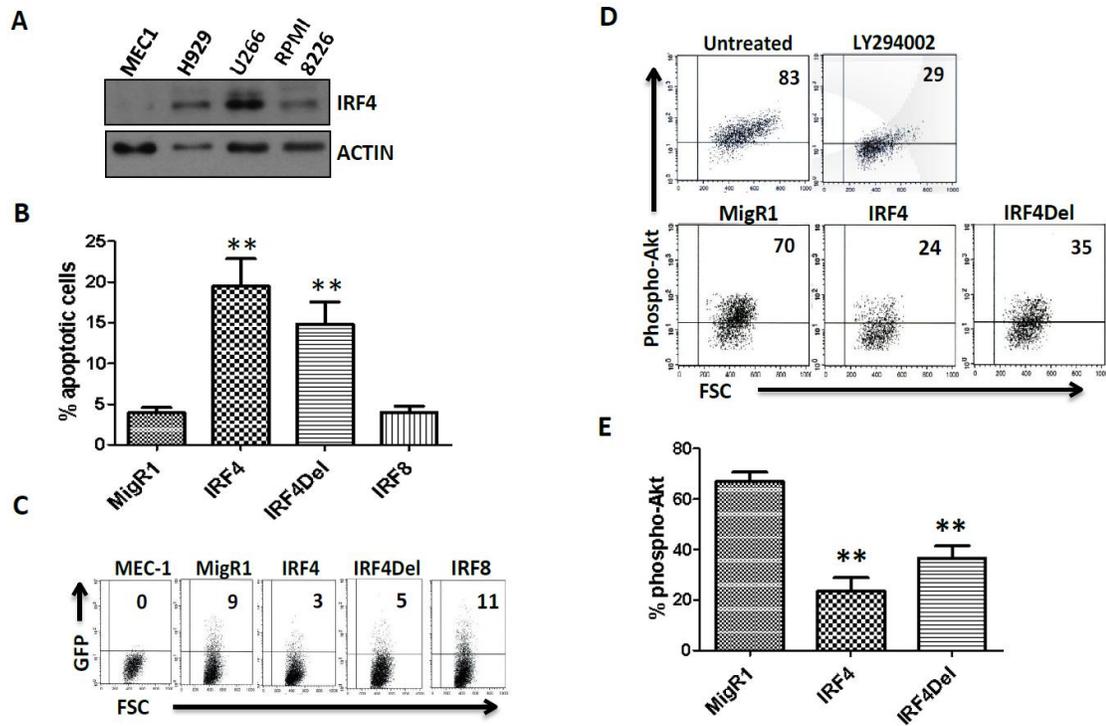


Figure 8 High levels of IRF4 inhibit AKT and promote apoptosis in human CLL cells. A) MEC-1 human CLL cells, H929, U266 and RPMI8226 multiple myeloma cells were lysed for Western blot analysis to detect IRF4 expression. B) MEC-1 cells were transfected with MigR1, MigIRF4, MigIRF4Del or MigIRF8 expression 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 were averages and SD of three independent experiments. ** $p < 0.01$. C) The percentage of GFP positive MEC-1 cells transfected with MigR1, MigIRF4, MigIRF4Del and MigIRF8. The numbers indicate the percentage of GFP+ cells. D) MEC-1 cells were transfected with MigR1, MigIRF4 and MigIRF4Del and 48h after transfection, phospho-Akt and GFP double positive cells were measured by FACS in each group. Additionally, MEC-1 cells were treated with PI3K inhibitor LY294002 (50 μ M). After two days, intracellular phospho-Akt was analyzed by FACS. The untreated cells were analyzed as control. The numbers are percentages of phospho-Akt positive cells. E) Averages and SD of three independent experiments described above. ** $p < 0.01$.

2.4 DISCUSSION

A previous GWAS 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 reduced level of IRF4 and the development of CLL. We used IRF4 heterozygous mutant NZB mice to mimic the effect of germline SNP variants in IRF4 gene and the associated low levels of IRF4 in CLL patients. Our results show that CLL development was dramatically accelerated in the NZB IRF4^{+/-} mice. The average onset of CLL in NZB mice is around 12 month, 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 human, our analysis reveals that spleen, but not lymph node and bone marrow, is the major site of the expansion for the NZB IRF4^{+/-} CLL cells. The expansion of human CLL cells occurs in the proliferation center found predominantly in lymph node 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 splenic microenvironment provides the much-needed pro-survival/proliferation signals in this mouse model. Human CLL cells, like murine B1 cells, possess poly-reactive BCRs that recognize self-antigen and microbial antigen and chronic autoantigen stimulation is believed to play a critical role in the development and progression of CLL (36, 37). Our result shows that NZB IRF4^{+/-} CLL cells are hyperresponsive to BCR stimulation, a property that would render them more sensitive to autoantigen stimulation *in vivo* and thus confer survival advantage for the cells. However, reconstitution of IRF4 expression in NZB IRF4^{+/-} CLL cells 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 pro-survival Bcl-2 family proteins. Among the Bcl-2 family members, Mcl-1 is the major pro-survival 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 the loss of negative regulators miR15a/16 (39, 40). Our results show that in the NZB IRF4^{+/-} CLL cells miR15a/16-1 are expressed at levels that are comparable to those in the NZB IRF4^{+/+} B cells, indicating that 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 the 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^{+/-} B1 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^{+/-} B1 cells are most likely due to 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^{+/-} B1 cells lead to expansion of precursor CLL cells and render them more susceptible to subsequent transformation events.

Besides regulating 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, raise 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 GWAS 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 redundantly fashion to control early stage of B cell development (15). However, it appears that IRF4 and IRF8 play opposite role in the development of CLL. Further analysis of IRF4 effect reveals that high levels of IRF4 suppress Akt activity. Akt activity is regulated by PI3 Kinase (PI3K) downstream of BCR signaling and 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). 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 BAD and caspase-9 (47, 48).

Since high levels of IRF4 in NZB IRF4^{+/-} CLL cells did not attenuate BCR signaling, the suppressive effect of IRF4 on Akt is unlikely a result of weakened BCR signaling in CLL cells. How Akt activity is suppressed by IRF4 is unclear. Intriguingly, our result shows 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 which shows that IRF4, without its DNA binding domain, can still suppress BCR/ABL oncogene induced myeloid leukemia (49). Their analysis further shows that it is the C-terminal IRF association domain that is critical for the tumor suppressive activity of IRF4. As DNA binding domain of IRF4 contains 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 directly interact 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, nevertheless, our findings presented here demonstrate that 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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CHAPTER 3

A role for IRF4 in the development of CLL

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3.1 INTRODUCTION

Interferon regulatory factor 4 (IRF4) is a transcriptional regulator of immune system development and function(1). In B cells, IRF4 is critical for their early stage development in the bone marrow as well as their functions in the periphery(2, 3). Previous works from our group have shown that IRF4 and, its closely related family member IRF8, orchestrate the transition from the large pre-B cell to the small pre-B cells by regulating cell cycle exit and by promoting light chain rearrangement(4-6). In mature B cells, IRF4 is essential for class-switching, germinal center exit and the differentiation into antibody secreting plasma cells(7-9). 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(10, 11). However, in multiple myeloma, IRF4 behaves as an oncogene to promote the survival of those cells(12).

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries and is characterized by a monoclonal accumulation of neoplastic CD5+ B cells in blood, bone marrow, and secondary lymphoid tissues (13). Multiple factors have been implicated in the molecular etiology of CLL, including genetic predisposition, autoantigen stimulation, microRNAs (miRNAs), and cytogenetic abnormalities(14). The molecular pathogenesis of CLL has not been fully elucidated, largely because few genetic abnormalities have been conclusively linked to the pathogenesis of CLL. A recent genome-wide SNP association study (GWAS) in CLL patients identified IRF4 as a major susceptible gene for CLL(15). Fine-scale mapping analysis identified four SNPs mapped to a 3-kb region in the 3'-UTR of the IRF4 gene(16). Further analysis of IRF4 expression in Epstein-Barr virus (EBV) transformed lymphocytes suggests that presence of the

SNPs was associated with a downregulation of IRF4(15). Low levels of IRF4 were also found to correlate with poor prognosis in CLL patients(17). Mutations in the DNA binding domain of IRF4 gene were identified in human CLL patients(18). It occurs only in 1.5% CLL cases with majority of the cases also carrying trisomy 12. It remains unclear whether and how these mutations impact the functions of IRF4.

We used a naturally occurring, low penetrance model of CLL, the New Zealand Black (NZB) mouse to study the role of IRF4 in CLL development (19). We bred the IRF4^{+/-} mice expressing low levels of IRF4 to the NZB mice (NZB IRF4^{+/-}), to study how low levels of IRF4 affects the pathogenesis of CLL in this model (19). Interestingly, our results showed that CLL development is markedly accelerated in NZB mice expressing low levels of IRF4 (19). NZB IRF4^{+/-} mice developed CLL at 4-5 months of age with 100% disease penetrance, when compared with the NZB IRF4^{+/+} mice that developed CLL at 10-12 months of age with 30% disease penetrance (19). The pathogenesis of CLL in NZB mice has been linked to multiple genetic lesions, one of which is a SNP identified in the 14q13 locus harboring miR15a/16-1 gene (20). The identified SNP interferes with the processing and functional maturation of the miRNA15a/16-1 that are known to be important for CLL (20). The 14q13 locus is syntenic to the 13q14 locus in humans, which is deleted in ~55-60% of CLL cases, most with indolent disease course (40, 45). Additionally, the deletion of this locus in its entirety or miRNAs alone leads to spontaneous CLL development at low penetrance in rodents (40, 45). Therefore, our studies show that low levels of IRF4 collaborate with the preexisting genetic defects in NZB mice to radically accelerate CLL development (19). However, the presence of preexisting genetic defects in the NZB mice precluded these studies from conclusively demonstrating a role for IRF4 in CLL initiation.

The cellular origin of human CLL remains unclear, however, CLL cells are believed to be derived from B1 cells in rodent(21). B1 cells are a small B cell subset that normally resides in the peritoneal (PC) and pleural cavities. B1 cells play an important role in host defense against microbial infection and are the major producers of natural antibodies in serum(22). B cell receptors (BCR) expressed on B1 cells are polyreactive with a very restricted immunoglobulin heavy chain (IgH) repertoire. Vh11 family of IgH gene is one of those unique Ig genes that are found only in B1 cells where it pairs with light chain to form the BCR that recognizes phosphatidylcholine (PtC) on senescent red blood cells (23). Analysis of Vh11 knock-in mice confirmed that B cell expressing Vh11 knock-in allele give rise to B1 cells, whose population is expanded dramatically in the Vh11 knock-in mice(24). Here, we report that IRF4 deficient mice expressing Vh11 knock-in allele (IRF4^{-/-}Vh11) spontaneously developed early onset CLL at 100% penetrance. Moreover, reconstitution of IRF4 expression in IRF4^{-/-}Vh11 CLL cells inhibited their survival.

3.2 MATERIALS AND METHODS

Mice. IRF4 deficient mice (IRF4^{-/-}) and Vh11 knock-in mice have been previously described(24, 25). The Rag2 and common gamma chain double deficient mice (Rag2^{-/-}γ^{-/-}) mice were obtained from Taconic. 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 University of Nebraska Medical Center. The mice age from 8 to 30 weeks were used for this study.

Transplantation of CLL cells. CLL cells were isolated from spleens of IRF4^{-/-}Vh11 mice via negative selection. The isolated CLL cells were injected via retro-orbital sinus into non-irradiated Rag2^{-/-}γ^{-/-}.

10^6 cells were used for each injection. The CLL cells were monitored weekly through blood analysis.

***In vivo* BrdU labeling.** The *in vivo* BrdU labeling assay was performed as described before(26). Mice were injected i.p. with 6 mg/ml BrdU (Sigma-Aldrich) and 12h later the cells were isolated for analysis. Three mice from each group were used for this assay. Cells from blood, lymph node and spleen were stained with antibodies against CD5, IgM and CD19. After fixation, the incorporated BrdU was detected with a BrdU flow kit (Pharmingen). The percentages of BrdU positive cells were detected by FACS analysis.

TUNEL and activated caspase 3 assays. The apoptosis status of CLL cells in mice were examined with terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. The TUNEL assay was conducted as previously described(11). The cells were isolated and stained with surface antibodies (CD5 and IgM). The TUNEL positive cells were revealed with an APO-direct kit (BD Pharmingen). The activated caspase 3 was also used to detect apoptotic cells. In this case, the assay was carried out with a kit from BD Pharmingen.

Transfection of CLL cells *in vitro*. CLL cells were isolated from spleens of IRF4^{-/-}Vh11 mice and cultivated on top of S17 stromal layer in media containing RPMI-1640 with 10% FBS. To reconstitute expression of IRF4, IRF4^{-/-}Vh11 CLL cells were mixed with either control vector or IRF4 expressing vector. 10×10^6 CLL cells and 20 μ g of plasmid were used for each transfection. The transfection was carried out in a Nucleofector (Lonza) with Solutioin R using program U-016. The transfected cells were analyzed 48 hours later.

Assay to measure miR15a/16-1.

Total RNA was extracted from the cells with a microRNA isolation kit (Ambion) and was converted to cDNA using Taqman MicroRNA Reverse Transcription kit and Taqman RT primers (ABI). For miRNA quantification, TaqMan miRNA assays (ABI) were used according to the manufacturer's protocol. Expression levels were normalized to the U6 snRNA.

Western Blot analysis. Splenic B cells were isolated via negative selection. The isolated cells were lysed and the lysates were separated by SDS-PAGE gel. The membranes were incubated with indicated antibodies and the signals were revealed with ECL detection system (Pierce). The antibodies against Tcl-1, Bcl-2, Bcl-xl and Mcl-1 were obtained from Cell Signaling.

3.3 RESULTS***3.3.1 Spontaneous CLL development in IRF4 deficient Vh11 knock-in mice.***

We have been using IRF4 deficient mice (C57B6) to examine the role of IRF4 in B cell development and function(3). However, we failed to observe an overt CLL development in these mice (data not shown). We reasoned that this may be caused by insufficient number of B1 cells in wild type background, as B1 cells consist of only 5% of total B lymphocytes in adult mice. Therefore, we postulated that the effect of low levels of IRF4 on CLL development should be examined in mice that have an expanded B1 cell population. Since Vh11 knock-in mice have an expanded B1 cell population, we decided to generate IRF4 deficient mice expressing Vh11 knock-in allele. To this end, we backcrossed Vh11 mice (CB17) into IRF4^{-/-} mice (C57B6) for at least six generations (IRF4^{-/-}Vh11). To monitor pathogenesis of CLL, we collected blood monthly from saphenous vein of IRF4^{-/-}Vh11, IRF4^{+/-}Vh11, and IRF4^{+/+}Vh11 littermate control mice. The appearance of a monoclonal IgM⁺CD5⁺ population and its percentage among peripheral blood mononuclear cells (PBMC) were

used for initial CLL diagnosis. The diagnostic criterion for CLL in IRF4^{-/-}Vh11 mice is the appearance of monoclonal IgM⁺CD5⁺ cells constituting over 20% of PBMC. If the percentage of the IgM⁺CD5⁺ clone is under 20% of the PBMC, the diagnosis is monoclonal B cell lymphocytosis (MBL).

Interestingly, 7 out of 12 IRF4^{-/-}Vh11 mice developed CLL after just 5 months while the rest developed MBL (Fig 1A). The mice with MBL all progressed into CLL within another 5 months. In contrast, none of the IRF4^{+/+}Vh11 control mice developed CLL or MBL within 12 month (Fig. 1A). We failed to observe overt CLL development in IRF4^{+/+}VH11 mice. However, two IRF4^{+/+}VH11 mice did develop MBL like disease at 10 months of age (Fig 1A). Among the CLL cases that emerged from IRF4^{-/-}Vh11 mice, 70% resembled indolent CLL where the mice had no systemic symptoms and histological analysis revealed little infiltration of CLL cells into non-lymphoid organs and tissues. The other 30% exhibited aggressive behavior where the mice exhibited systemic symptoms accompanied by infiltrations of CLL cells into non-lymphoid organs and tissues. The mice in the latter case often succumbed to diseases within 12 months (Fig 1B). FACS analysis was carried out to characterize CLL cells in the IRF4^{-/-}Vh11 mice (Fig. 1C). IgM⁺CD5⁺ CLL cells were detected in blood (BL), bone marrow (BM), lymph node (LN) and spleen (SPL) of IRF4^{-/-}Vh11 mice (Fig. 1C). In Peritoneal Cavity (PC) where B1 cells normally reside, the IgM⁺CD5⁺ B1 cells were also detected in the IRF4^{+/+}Vh11 control mice. The presence of significant numbers of CLL cells in LN and SPL of IRF4^{-/-}Vh11 mice caused massive splenomegaly and lymph adenopathy. The splenomegaly and lymph adenopathy were more prominent in IRF4^{-/-}Vh11 mice with CLL than those with MBL. The average numbers of IgM⁺CD5⁺ CLL cells in spleens of IRF4^{-/-}Vh11 mice with CLL and MBL were $2.4 \pm 1.2 \times 10^8$ (n=6) and $0.6 \pm 0.4 \times 10^8$ (n=5), respectively. To understand the progression of CLL cells in IRF4^{-/-}Vh11 mice, we calculated the frequencies of CLL cells among PBMC over a period of eight months. The IgM⁺CD5⁺ CLL cells typically started to appear in blood

of IRF4^{-/-}Vh11 mice at 2 to 4 months of age (Fig. 1D). The frequencies of the CLL cells steadily increased over the next few months, reaching an average of 69% of PBMC at 8 months of age. In contrast, the frequencies of IgM+CD5+ cells were low and relatively stable in IRF4^{+/+}VH11 and IRF4^{+/-}VH11 mice throughout the entire period (Fig. 1D).

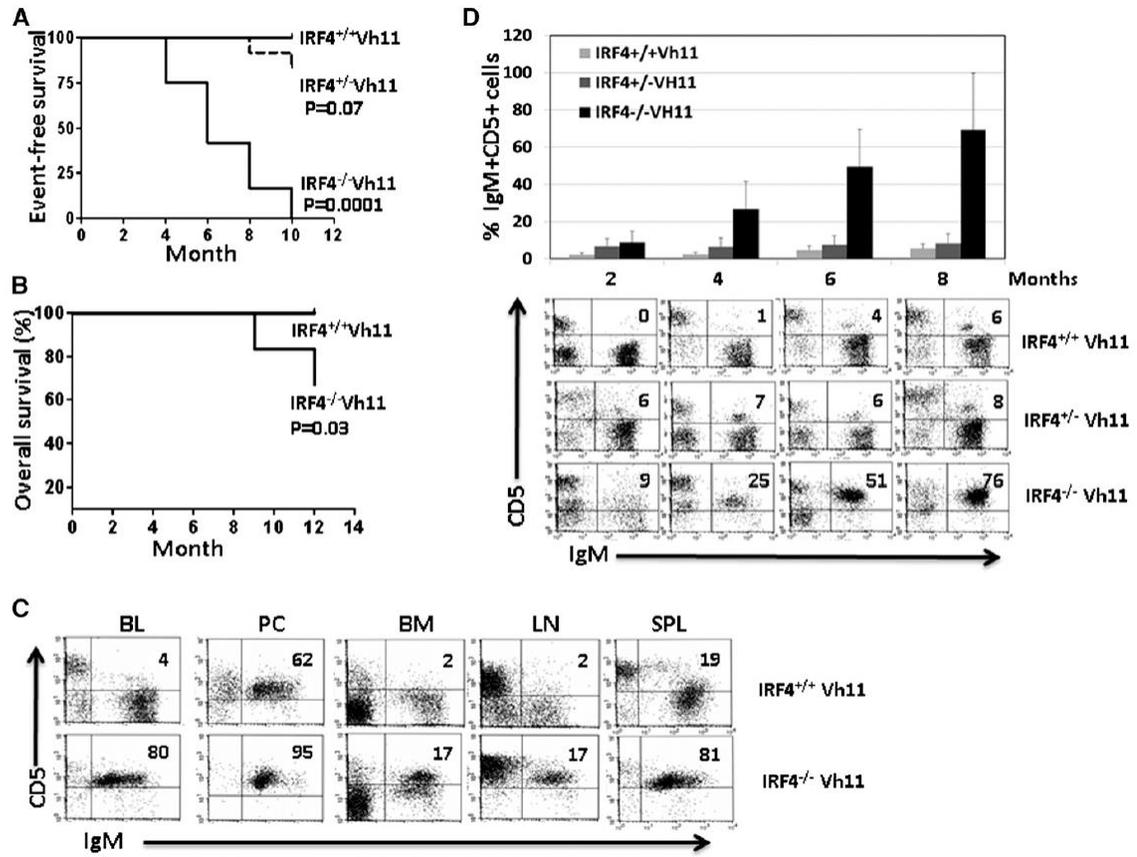


Figure 1 Spontaneous CLL development in IRF4 deficient Vh11 knock-in mice. A) Kaplan-Meier Event-free survival curve of IRF4^{+/+}Vh11, IRF4^{+/-}Vh11, and IRF4^{-/-}Vh11 mice. 12 mice from each genotype were used for the analysis. P values are pair-wise comparison (log rank test) between IRF4^{+/+}Vh11 and IRF4^{+/-}Vh11, and between IRF4^{+/+}Vh11 and IRF4^{-/-}Vh11. Graphpad PRISM was used to plot the survival curve and to calculate p value. B) Kaplan-Meier Overall Survival curve of IRF4^{+/+}Vh11 and IRF4^{-/-}Vh11 mice. 10 mice from each genotype were used for the analysis. C) Cells were isolated from blood (BL), PC, bone marrow (BM), lymph node (LN) and spleen (Spl) of five-months old IRF4^{+/+} Vh11 and IRF4^{-/-}Vh11 mice. The isolated cells were stained with antibodies against CD5 and IgM and analyzed by FACS. D) Blood were collected from IRF4^{+/+}Vh11, IRF4^{+/-}Vh11, and IRF4^{-/-}Vh11 mice for FACS analysis. The blood were collected from these mice every two months for a period of eight months. There were five mice in each group. The frequency of CD5+IgM+ cells among PBMC was calculated by FACS analysis. The average and SD of the frequency of CD5+IgM+ cells in each group at different time points were calculated (Fig. 1D top). A representative FACS analysis for each group at different time points was shown (Fig. 1D bottom).

3.3.2 Phenotypical and Histological analyses of CLL cells in IRF4^{-/-}Vh11 mice.

To further characterize CLL cells, we isolated splenic cells from IRF4^{-/-}Vh11 and IRF4^{+/+}Vh11 mice and stained them with a panel of antibodies against CD19, B220, CD23, CD21, IgD, CD1d, IgMa and IgMb. CD23, CD21 and IgD are expressed at high levels on Follicular B cells (major splenic B cell population) but at low levels on B1 cells. Marginal zone B cells express high levels of CD1d whereas other B cell subsets including B1 cells express intermediate level of CD1d (CD1d^{int}). The knock-in Vh11 heavy chain is IgMa allotype which can be distinguished from endogenous Ig heavy chain (IgMb). When compared to splenic B cells in IRF4^{+/+}Vh11 mice, IRF4^{-/-}Vh11 CLL cells were CD19⁺, B220^{low/-}, CD23⁻, CD21⁻, IgD^{low} and CD1d^{int} (Fig. 2A). The surface phenotype of IRF4^{-/-}Vh11 CLL cells resembles B1 cells from which they are derived. Finally, IRF4^{-/-}Vh11 CLL cells were IgMa⁺IgMb⁻, indicating that they express only Vh11 knock-in allele. In contrast, B cells in IRF4^{+/+}Vh11 mice were a mixed populations where some B cell expressed the knock-in allele (IgMa⁺) while others expressed the product of rearranged endogenous heavy chain (IgMb⁺).

IRF4^{-/-}Vh11 mice exhibited splenomegaly and in cases of aggressive CLL, enlarged livers. These findings indicate that CLL cells infiltrate not only lymphoid organs and tissues but also nonlymphoid organs including livers. To verify this result, we performed histological analysis of spleens and livers of IRF4^{-/-}Vh11 and IRF4^{+/+}Vh11 mice. Histological examination of spleen of IRF4^{-/-}Vh11 mice revealed a grossly distorted white pulp and red pulp, with much larger, irregular lymphoid follicles (Fig.2B). H&E staining of liver revealed infiltration of lymphocytes around central vein in the IRF4^{-/-}Vh11 mice (Fig.2B). Although the perivascular infiltrations were prominent in the liver of IRF4^{-/-}Vh11 mice, infiltrating lymphocytes could also be detected in other regions of the liver.

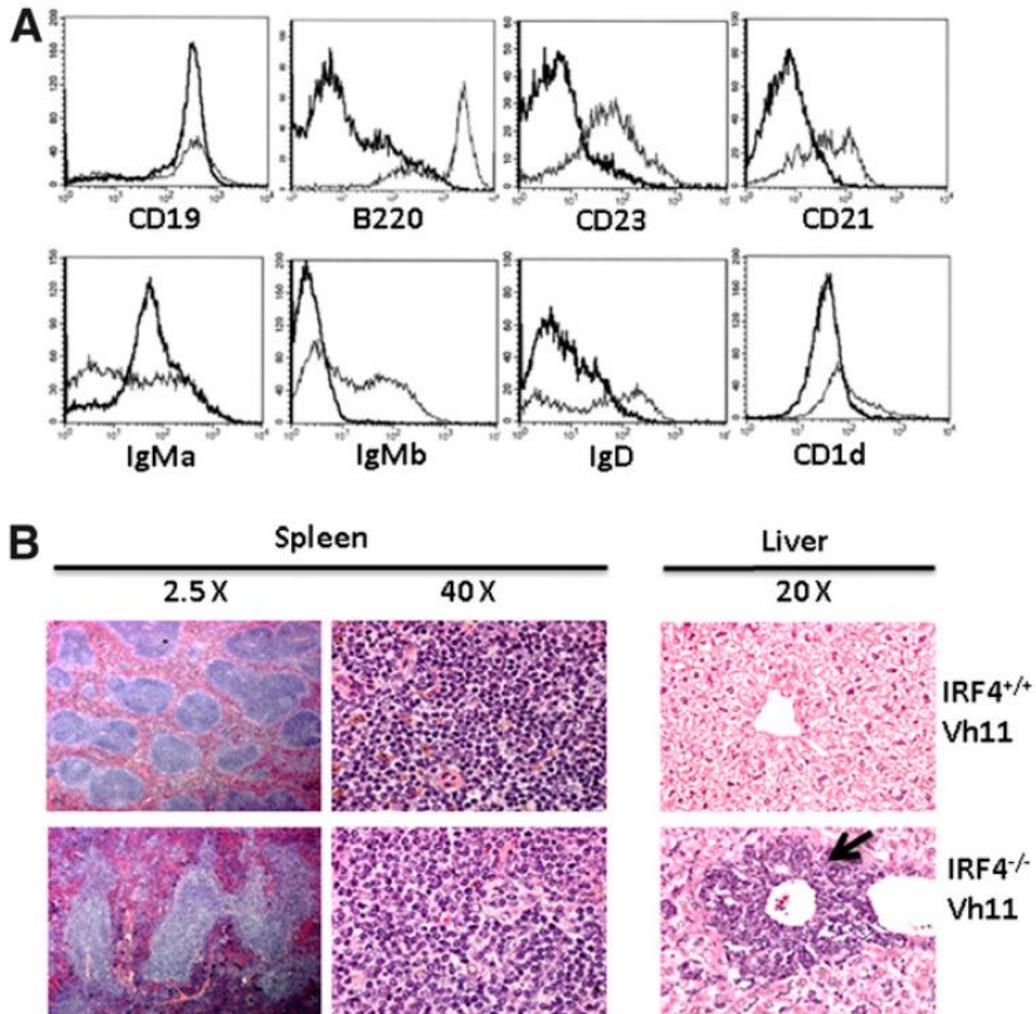


Figure 2 Phenotypal and histological analyses of CLL cells in the IRF4^{-/-}Vh11 mice. A) Splenocytes from IRF4^{+/+} Vh11 and IRF4^{-/-}Vh11 mice were stained with antibodies against CD19, B220, IgMa, IgMb, CD21, CD23, IgD and CD1d. The stained cells were analyzed by FACS. The data were presented as histogram under a B cell gate. Dark line: IRF4^{-/-} Vh11 CLL cells; light line: IRF4^{+/+} Vh11 B cells. B) Spleens and livers were isolated from five-month old IRF4^{-/-}Vh11 and IRF4^{+/+}Vh11 mice. The tissues were fixed, sectioned, stained with hematoxylin and eosin (H&E). The spleen sections are shown at 2.5x and 40x; the liver sections are shown at 20x. Arrow indicates the infiltrated CLL cells in liver.

3.3.3 IRF4^{-/-}VH11 CLL cells proliferate mainly in spleen and are resistant to apoptosis.

We wanted to further characterize the behavior of IRF4^{-/-}Vh11 CLL cells by examining their proliferation and survival *in vivo*. To determine their proliferating rate, IRF4^{-/-}Vh11 mice were pulse-labeled with BrdU and the percentages of BrdU positive IgM⁺CD5⁺ cells in blood, spleen and lymph node were examined. IRF4^{+/+}Vh11 mice were examined as control. In blood, the percentages of BrdU positive cells in IRF4^{+/+}Vh11 and IRF4^{-/-}Vh11 mice were at 1.1% and 1.3%, respectively (Fig.3A). The low percentage of BrdU positive cells in blood indicates that IRF4^{-/-}Vh11 CLL cells do not proliferate in blood. Similarly, only 2% of IRF4^{-/-}Vh11 CLL cells were found to be BrdU positive in lymph node, indicating that Lymph node is not the major site for their expansion either. In contrast, 4.8% of splenic CLL cells in IRF4^{-/-}Vh11 mice were stained positive for BrdU, indicating that spleen is the major organ for their expansion. To assess apoptotic status of IRF4^{-/-}Vh11 CLL cells, we performed TUNEL assay on isolated splenic CLL cells. While 4.5±1.12% of IgM⁺CD5⁺ splenic B1 cells were found to undergo apoptosis in IRF4^{+/+}Vh11 mice, only 0.2±0.04% of IgM⁺CD5⁺ CLL cells in IRF4^{-/-}Vh11 mice were TUNEL positive, indicating that IRF4^{-/-}Vh11 CLL cells are resistant to apoptosis (Fig.3B). In addition, very few TUNEL positive CLL cells were detected in blood, lymph node and bone marrow (data not shown). In summary, these results show that expansion of IRF4^{-/-}Vh11 CLL cells mainly occurs in the spleen and the CLL cells are resistant to apoptosis.

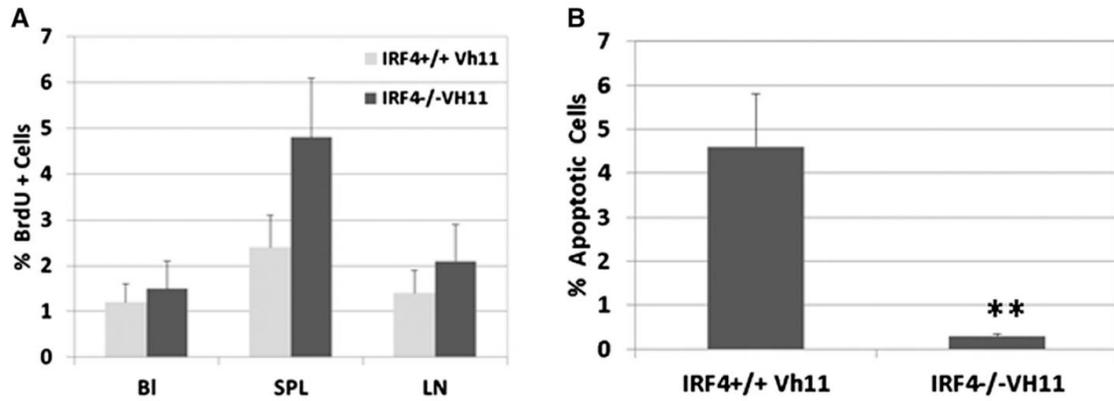


Figure 3 IRF4^{-/-}Vh11 CLL cells proliferate mainly in spleen and are resistant to apoptosis. A) To characterize the proliferation of IRF4^{-/-}Vh11 CLL cells in vivo, we pulse-labeled the mice with BrdU. 12h later, cells were isolated from blood, spleen and lymph node and stained with antibodies against CD5, IgM and BrdU. The BrdU positive cells were revealed by FACS analysis. B) splenocytes were isolated from IRF4^{-/-}Vh11 and IRF4^{+/+}Vh11 mice and stained with CD5 and IgM. The apoptotic cells were detected by TUNEL assay. Values are averages and standard deviations of three independent experiments. * p<0.01

3.3.4 IRF4^{-/-}Vh11 CLL cells are transplantable in immunodeficient host mice.

To determine whether the CLL cells in the IRF4^{-/-}Vh11 mice are transplantable, we isolated splenic CLL cells from IRF4^{-/-}Vh11 mice and transplanted them into immunodeficient Rag2^{-/-}gamma^{-/-} host mice. The presence of IgM⁺CD5⁺ CLL was detected in the blood of host mice within a week of transplantation and the host mice (n=10) all succumbed to CLL within one month post-transplantation. Three weeks after transplantation, CLL cells in the blood and spleen of host mice were analyzed by FACS (Fig. 4A). By this time, 95% of PBMC consisted of IgMa⁺ CD5⁺ CLL cells. The spleens of host mice were dramatically enlarged where 85% of splenic cells were CLL cells. H&E staining of the spleen revealed that the spleens had no discernible white and red pulps and CLL cells were evenly distributed throughout spleen (Fig. 4B). Similar to the donor CLL cells, transplanted CLL cells were resistant to apoptosis (Fig 4C). BrdU labeling study revealed that transplanted CLL cells are proliferating predominantly in spleen: 16 % of transplanted CLL cells in spleen were BrdU⁺ while only 4% of them were BrdU positive in blood. Collectively, our results show that IRF4^{-/-}Vh11 CLL cells are transplantable in immunodeficient host mice

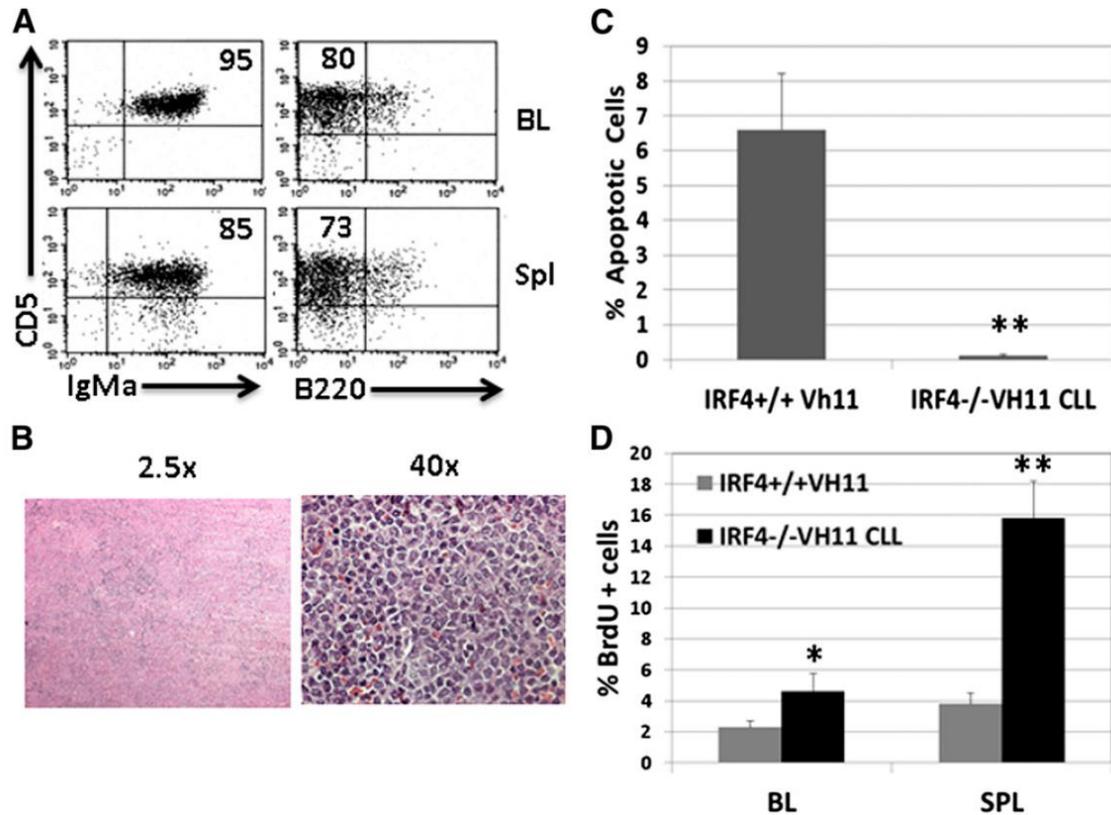


Figure 4 IRF4^{-/-}Vh11 CLL cells are transplantable in immunodeficient host mice. CLL cells were isolated from spleens of IRF4^{-/-} Vh11 mice and transplanted into Rag2^{-/-}γ^{-/-} mice. 10⁶ CLL cells were used for each injection. Three weeks after transplantation, the mice were analyzed. A) Blood (BL) and spleen (Spl) of the host mice were analyzed by FACS. B) H&E staining of spleen of transplanted host mice. C) CLL cells were isolated from spleens of host mice. TUNEL assay was used to detect apoptotic cells. Splenic B cells from IRF4^{+/+}Vh11 mice were analyzed as control. D) Host mice were pulse-labeled with BrdU and examined 12h later. BrdU+ cells in blood and spleen of IRF4^{+/+}Vh11 mice were also examined and used as control. * p<0.05, ** p<0.01.

3.3.5 Molecular signatures of IRF4^{-/-}Vh11 CLL cells.

To further characterize IRF4^{-/-}Vh11 CLL cells, we examined some common molecular signatures that are associated with human CLL cells, including expression of Bcl-2 family members, T cell leukemia/lymphoma 1 (Tcl1) and miR15a/16-1 microRNAs. Splenic CLL cells were isolated from five IRF4^{-/-}Vh11 mice and lysed. As controls, we also isolated splenic B cells from two IRF4^{+/+}Vh11 (Vh11) mice and one IRF4^{+/+} mice (B6). Expression of Bcl-2 family members Bcl-2, Bcl-xl and Mcl-1 was examined by Western blot (Fig. 5A). Our results show that compared to controls, expression of Mcl-1 was significantly elevated in all five CLL samples (Fig.5A). In contrast, expression of Bcl-2 was decreased in majorities of the CLL samples. Expression of Bcl-xl was moderately increased in some but not all CLL samples. Interestingly, compared to B cells in IRF4^{+/+} mice, expression of Bcl-2 family members appears to be elevated in splenic B cells of IRF4^{+/+}Vh11 mice. The reason behind this observation is not clear. Since expression of Bcl2, Mcl-1 and Bcl-xl can be induced by BCR signaling, it is possible that IRF4^{+/+}Vh11 B cells were in an activated state due to the presence of high levels of PtC in spleen. Expression of Tcl-1 can be detected in 90% human CLL cases and is found to be overexpressed in patients with aggressive CLL(27). Moreover, mice engineered to overexpress Tcl1 oncogene in B cells (E μ Tcl-1) develop late onset, aggressive CLL(28). However, except for CLL cells isolated from E μ Tcl-1 transgenic mice, expression of Tcl1 could not be detected in samples derived from IRF4^{-/-}Vh11 CLL cells, indicating that expression of Tcl1 was very low in those samples (Fig. 5A). Additionally, compared to their expressions in the control cells, miR15a/16-1 expression were slightly elevated in the CLL cells (Fig.5B). In summary, our results show that expression of Mcl-1 is significantly elevated in the IRF4^{-/-} Vh11 CLL cells, however, Tcl1 and miR15a/16-1 expression are not deregulated in these cells.

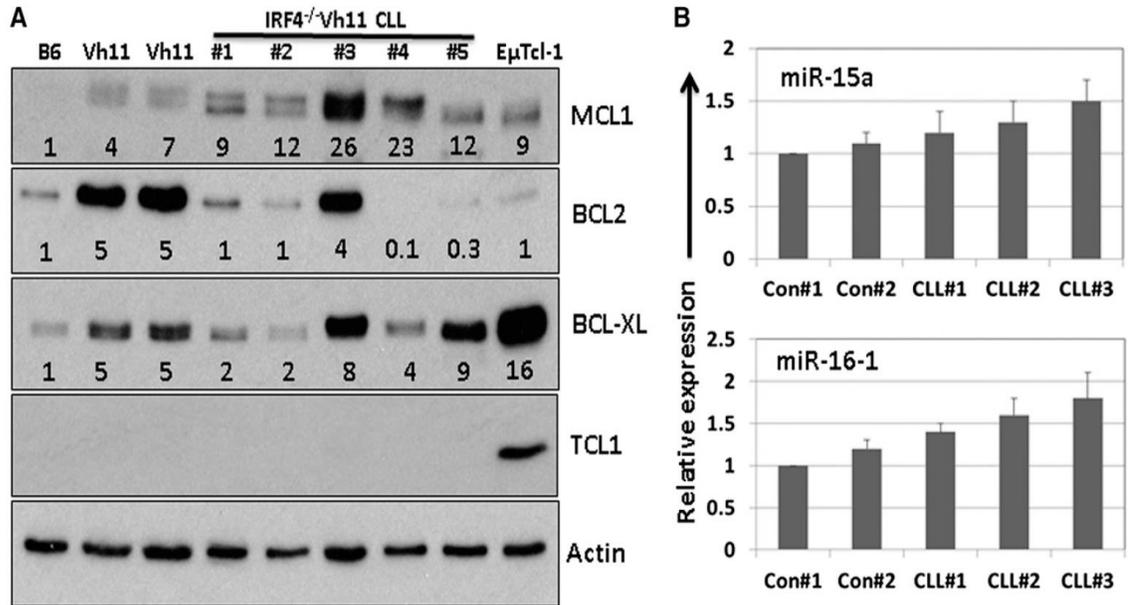


Figure 5. Molecular characterization of IRF4^{-/-}Vh11 CLL cells. A) Splenic CLL cells were isolated from five IRF4^{-/-}Vh11 mice via negative selection and lysed for Western blot analysis with indicated antibodies. Splenic B cells from IRF4^{+/+}Vh11(Vh11) and IRF4^{+/+} (B6) mice were also isolated and analyzed as controls. Additionally, splenic CLL cells from E μ Tcl1 transgenic mice were used as 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 to controls (IRF4^{+/+}Vh11).

3.3.6 Reconstitution of IRF4 expression inhibits the survival of IRF4^{-/-}Vh11 CLL cells.

Since IRF4^{-/-}Vh11 CLL cells are resistant to apoptosis, we wanted to examine how IRF4 affects the survival of these CLL cells. To this end, we reconstituted the expression of IRF4 in the IRF4^{-/-}Vh11 CLL cells. Briefly, IRF4 expressing plasmid (co-expressing GFP) was transfected into cultivated IRF4^{-/-}Vh11 CLL cells using a Nucleofector. The effect of IRF4 reconstitution on CLL cells was examined after 48h. The control vector transduced CLL cells were analyzed as control. The transfection efficiency was around 10% for control transfected cells but only 3% in the IRF4 transduced cells. The apoptotic cells were detected with antibody against activated Caspase 3. Results of three independent experiments were shown (Fig. 6A). The averages and standard deviations of the three independent experiments were statistically analyzed (Fig. 6B). While the percentage of apoptotic cells in control transduced cells was only 2.3±0.6%, the percentage of apoptotic cells increased to 8.0±2.4% in the IRF4 transduced cells (Fig.6B). This result indicates that reconstitution of IRF4 promotes apoptosis in the IRF4^{-/-}Vh11 CLL cells *in vitro*.

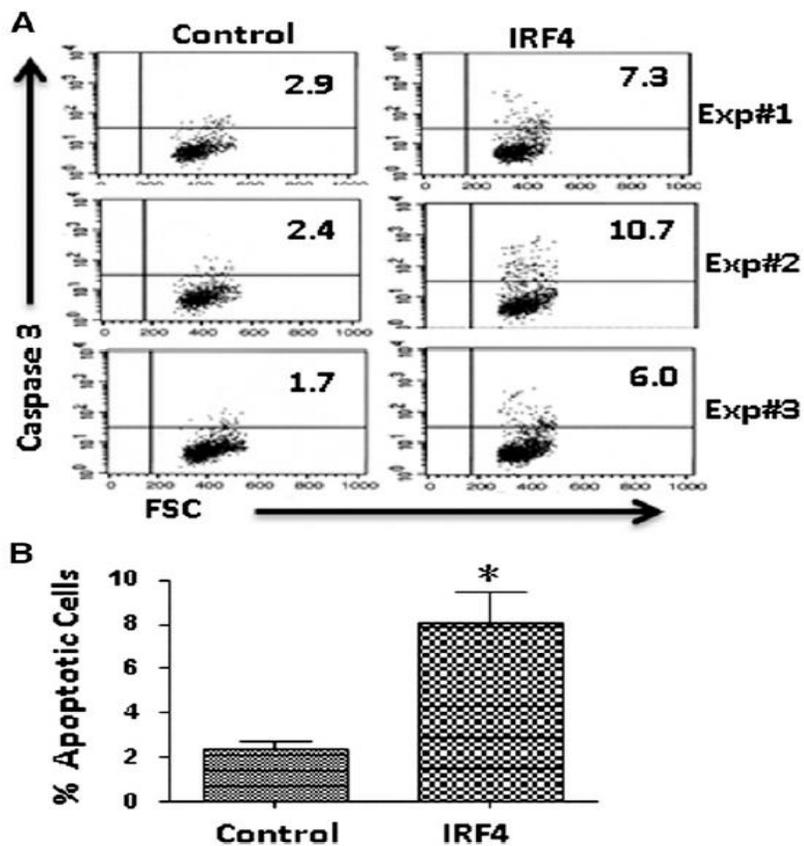


Figure 6 Reconstitution of IRF4 expression inhibits the survival of IRF4^{-/-}Vh11 CLL cells. A) CLL cells were isolated from spleen of IRF4^{-/-}Vh11 mice and plated on top of S17 stromal cells in RPMI-1640 media containing 10% FBS. IRF4 expressing vector and control vector were transfected into cultivated CLL cells using a Nucleofector. 48h later, the apoptotic cells in successfully transfected CLL cells (GFP+) were analyzed with a kit detecting activated Caspase 3 (BD Pharmingen). The results of three independent experiments were shown. B) Averages and standard deviations of three independent experiments. * p<0.02.

3.4 DISCUSSION

The SNPs in the 3'UTR of IRF4 gene locus were identified as a risk allele for both sporadic and familial CLL and at least one copy of the allele is present in over 86% CLL cases(29). 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 IRF4^{-/-}Vh11 mice develop early onset CLL at 100% penetrance supports this assertion. In this study, we used IRF4 deficient mice to mimic the effect caused by germline associated SNPs in CLL patients. However, IRF4 germline deficient mice harbor developmental defects not only in B cells but also in other lineages of immune cells including T cells(1). CLL development and progression can be regulated by both B cell intrinsic and extrinsic factors(30). A recent study has further demonstrated that survival and expansion of transplanted human CLL cells are dependent on autologous T cells(31). However, our result shows that IRF4^{-/-}Vh11 CLL cells were transplantable in immunodeficient host mice, indicating that survival and expansion of IRF4^{-/-}Vh11 CLL cells in the host mice are not dependent on other IRF4 deficient immune cells.

Our results revealed that spleen is the major organ where IRF4^{-/-}Vh11 CLL cells proliferate. It was initially thought that CLL cells have low proliferation index. However, heavy water experiment has demonstrated that a small fraction of CLL cells are actively cycling and about 2% of CLL cells are newly generated each day(32). In human, CLL cells proliferate in a unique structure called the proliferation center found predominantly in lymph node and bone marrow. Human CLL cells, like murine B1 cells, possess polyreactive BCRs that recognize self-antigen and microbial antigen and chronic autoantigen stimulation is believed to play a critical role in the development and progression of CLL.(33, 34) Interestingly, PtC was also identified as a common antigen recognized by many CLL clones derived from E μ Tcl-1 mice(28). Moreover, a recent study further demonstrates that autoantigen PtC promotes CLL progression by selecting variants with enhanced

BCR signaling(35). The reason that IRF4^{-/-}Vh11 CLL cells were found to proliferate predominantly in spleen could be due to the abundance of their cognate antigen there.

Our results show that IRF4^{-/-} Vh11 CLL cells express high levels of Mcl-1. Human CLL cells are known to overexpress Mcl-1 as well as other members of pro-survival Bcl-2 family proteins. Among the Bcl-2 family members, Mcl-1 is the major pro-survival factor for CLL cells(36). Clinically, Mcl-1 is shown to be better than other Bcl-2 family members at predicting prognosis and clinical behavior of CLL patients(37). MiR15a/16 were initially thought to inhibit CLL development mainly by suppressing Bcl-2 expression(38). However, recent genetic analysis indicates that miR15a/16 also regulate a group of proteins that are critical for cell cycle progression(39). Our results show that expression of miR15a/16-1 is not deregulated in the IRF4^{-/-} Vh11 CLL cells. Expression of miR15a/16-1 also appears to be normal in the Tcl-1 transgenic mice. Only in Tcl-1 mice that are null for p53, expression of miR15a/16-1 was found to be dramatically reduced(40). High levels of Tcl-1 activate Akt and promote survival of CLL cells(41, 42). However, our results show that Tcl-1 is expressed at low level in IRF4^{-/-} Vh11 CLL cells.

Several mouse models have been generated to mimic pathogenesis of human CLL. E μ Tcl-1 transgenic mice develop lymphoproliferative diseases at 100% penetrance(43). CLL cells in E μ Tcl-1 transgenic mice possess stereotype BCR and resemble aggressive human CLL(28). In this model, CLL cells can be detected at 7 to 8 months of age and the all mice eventually succumb to disease. Although Tcl-1 is overexpressed in subset of human CLL patients, E μ Tcl-1 transgenic mice are not associated with any genetic lesion commonly associated with human CLL. Deletion of 13q14 is detected in 50 to 60% of human CLL cases(14). Recent efforts to mimic this genetic lesion in mice led to generation of three mice lines: 1) the first mice line targeting only miR15a/16-1 located in

the intron of Dleu2; 2) the second mice line targeting minimal deletion region (MDR) containing miR15a/16-1 and the entire Dleu2 gene; 3) the third mice targeting the common deleted region (CDR) including MDR region and beyond(39, 44). All three mice lines develop late onset lymphoproliferative disease resembling indolent CLL. The severity of the diseases is proportional to the length of deleted genetic region. The disease penetrance in the three lines also varies: 67% for CDR mice, 42% for MDR mice and 26% for miR15a/16-1 deletion mice. The 13q14 deletion models mimic a major genetic lesion in human CLL and are clinically relevant models to study pathogenesis of human CLL. However, whether this model is suitable for therapeutic purposes is unclear.

Our findings presented here establish IRF4^{-/-}Vh11 mice as a novel mouse model of CLL. IRF4^{-/-}Vh11 mice have the following unique features: 1) IRF4^{-/-}Vh11 mice carry a well-defined BCR which recognizes autoantigen PtC. It is worth pointing out that Vh11/Vk14 is a relevant BCR because anti-PtC IgMs have been found not only in normal individuals but also in CLL patients as well as patients with systemic lupus erythematosus(45-47); 2) IRF4^{-/-}Vh11 mice mimic a predominant genetic predisposition to CLL and thus represent a clinically relevant CLL model. 3) In contrast to other models, IRF4^{-/-}Vh11 mice develop early onset CLL with shortened disease latency; and 4) IRF4^{-/-}Vh11 mice develop a broad spectrum of lymphoproliferative diseases, from MBL, to indolent CLL and to aggressive CLL. Therefore, IRF4^{-/-}Vh11 mice will be a useful model to dissect molecular pathogenesis of CLL. Epigenetic changes have been linked to CLL development and progression(48, 49). It would be interestingly to study the epigenetic changes that accompany CLL initiation and progression in the IRF4^{-/-}Vh11 mice. In this study, IRF4^{-/-}Vh11 mice were generated through backcrossing Vh11 mice in the CB17 background to IRF4^{-/-} mice in the C57B6 background. The IRF4^{-/-}Vh11 mice used in this study have been backcrossed for at least six generations which

should contain over 98% of C57B6 DNA. Ideally, Vh11 should be backcrossed to C57B6 background for at least 10 generations to rule out the potential effect of a mixed genetic background on the development of CLL. Whether IRF4^{-/-}Vh11 mice would be useful for testing therapeutic drugs for CLL is unclear. In the future, in vivo evaluation with therapeutic agents such as Ibrutinib (Btk inhibitor) and Fostamatinib (Syk inhibitor) should be done to determine their effectiveness in this model.

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CHAPTER 4

Notch2 is Critical for CLL development in IRF4^{-/-}Vh11 mice.

4.1 INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is a clinically heterogeneous B cell malignancy that represents the most common adult leukemia in the western hemisphere. Despite considerable progress in our current understanding of CLL, the molecular events underlying the complex pathogenesis of CLL have not been fully elucidated. Recent Whole Genome Sequencing (WGS) studies have provided valuable insights into the molecular pathways deregulated during the pathogenesis of CLL. Intriguingly, multiple WGS studies have identified mutational activation of Notch signaling pathway as one of the most recurrent molecular event in human CLL (1-5). Moreover, the CLL patients carrying mutations in Notch signaling pathway have poor clinical outcomes and an increased tendency towards Richter transformation to Diffused Large B cell Lymphoma (DLBCL) (1, 2, 6). In CLL patients, Notch signaling pathway can be activated by mutations that primarily affect the stability of Notch1 protein (2, 5). Notch mutations in CLL patients cause frameshift deletions leading to generation of protein without the PEST domain (2, 5). The PEST domain in Notch proteins are functionally important for ubiquitination and degradation of Notch proteins and hence for limiting the cellular responses to Notch signaling (7). Other than the mutational activation, studies have also reported constitutively high expression of Notch1 and Notch2 leading to activation of Notch signaling in human CLL cells (8). *In vitro* studies relying on co-culture of CLL cells with stromal cells have provided evidence for a role of Notch signaling in promoting the survival and chemo-resistance of CLL cells (9, 10). Although, these studies have linked aberrant Notch signaling to the pathogenesis of CLL *in vitro*, whether Notch signaling is critical for CLL development *in vivo* remains unknown. Furthermore, the molecular pathways that lead to the deregulated Notch signaling in CLL cases without Notch mutations are still poorly defined.

Interferon Regulatory Factor 4 (IRF4) belongs to the IRF superfamily of transcription factors and regulates multiple developmental stages and functional processes in B lymphocytes. IRF4 functions redundantly with its homologous family member IRF8 at the early stages of pre-B cell differentiation (11). In mature B cells, IRF4 is required for terminal differentiation to plasma cells (12, 13). In distinct B cell malignancies, IRF4 has been shown to possess both tumor suppressive and pro-oncogenic functions (14, 15). Recent studies from our group and others have established an important role of IRF4 in the development of CLL (16-18). A Genome Wide Association (GWA) study linked single nucleotide polymorphisms (SNPs) in the 3' untranslated region of *irf4* gene locus to the development of CLL (16). The SNPs in the IRF4 locus were reported to be present in majority of human CLL patients (86%) and were linked to downregulation of IRF4 mRNA (16, 19). Using distinct mouse models we have recently established a causal link between low levels of IRF4 and CLL development (17, 18). Vh11 knock-in (KI) mouse is a genetically engineered mouse which expresses a prearranged immunoglobulin heavy chain gene family Vh11. B cells expressing Vh11 heavy chain predominantly develops into a specialized B cell subset known as B1 cells that are also the presumed precursors of CLL cells in rodents (20). Remarkably, our studies revealed that IRF4 deficient Vh11 KI (IRF4^{-/-}Vh11) mice developed spontaneous CLL at complete penetrance (18). In contrast, neither the IRF4 deficiency nor the Vh11 KI background alone in mice led to CLL development (18). New Zealand Black (NZB) mouse is a well-described CLL model (21). Interestingly, our studies showed that low levels of IRF4 led to dramatically accelerated CLL development in NZB mice (17). Although our studies have established a causal relationship between low levels of IRF4 and CLL development, the molecular mechanism through which IRF4 suppresses CLL development remains unknown.

Interestingly, a recent study described expansion of a specialized mature B cell subset known as Marginal Zone B cells (MZ B cells) in IRF4 deficient mice (22). Moreover, the expansion of MZ B

cells were attributed to higher levels of Notch2 receptor and associated Notch signaling in the IRF4 deficient mature B cells (22). Although the precise mechanism through which IRF4 regulates Notch signaling remains unclear, this study identified IRF4 as a potential novel regulator of Notch signaling in mature B cells. Given the possible connection between Notch signaling and CLL development, we hypothesized that in the IRF4^{-/-}Vh11 mice Notch signaling is also deregulated and the deregulation plays a critical role in CLL development. IRF4^{-/-}Vh11 mouse is regarded as a novel mouse CLL model because it mimics a predominant genetic predisposition to CLL (23). Therefore, IRF4^{-/-}Vh11 mice are very useful in understanding not only the molecular mechanism through which IRF4 controls CLL development but also the pathogenesis of CLL in general. In the present studies we examined the role of Notch signaling in the development of CLL in IRF4^{-/-}Vh11 mice.

4.2 MATERIALS AND METHODS

Animal Studies. IRF4^{-/-}Vh11 mice were generated and monitored for CLL development as previously described (18). ERT cre, Notch2 floxed (24), Rosa-rtTA (25) and CD19cre (26) mice were generated as described previously and purchased from Jackson laboratory. NOD-scid gamma chain deficient mice were obtained from Jackson laboratory. TRE IRF4 transgenic mice were generated and treated with doxycycline as previously described (27). All experiments were performed according to the guidelines from National Institute of Health and with an approved protocol from Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Western Blotting. B cells and CLL cells from spleen were isolated by negative selection using MACS columns. Lysates were prepared and resolved using SDS-PAGE. The membranes were incubated with the indicated antibodies and Horse radish peroxidase (HRP) conjugated secondary

antibodies. The signals were generated using Enhanced Chemi-Luminescence (ECL) substrate solution from Thermo-Pierce. The antibodies against Notch1, Notch2, and Hes1 were purchased from Cell signaling Technologies. Direct HRP conjugated antibody against Beta Actin was purchased from Sigma.

Flow cytometry and cell sorting. The cells were isolated from respective tissues and pre-incubated with Fc-Block antibody. Flow cytometry staining was performed by incubation of cells with optimal amounts of biotinylated or directly fluorophore conjugated antibodies. Antibodies against mouse B220, IgM and CD5 proteins were purchased from BD-pharmingen. Anti-mouse Notch2 and the corresponding isotype control antibodies were purchased from Biolegend. Fluorescence activated cell sorter (FACS) analysis was performed using FACSCalibur flow cytometer. Cell sorting was performed using BD FACSAria flow cytometer.

Tamoxifen Treatment. 50 mg Tamoxifen was dissolved in 1ml ethanol and diluted to 10ml in cornoil. NSG mice were given 3 tamoxifen injections intraperitoneally of 2 mg each on three consecutive days.

Primary and Cell Cultures. All primary cells and Mec-1 CLL cell line were cultured in RPMI-1640 media containing 10% fetal bovine serum, 50 μ M Beta mercaptoethanol, 2mM L-glutamine and 100 U of penicillin and streptomycin. The B1 cells were isolated from peritoneal ascites following incubation in the tissue culture dishes for 6 hours to remove adherent macrophages.

Proliferation and Survival Assays. Proliferation of cells was revealed by Bromodeoxyuridine (BrDU) incorporation assay. Cells were incubated in 10 μ M BrDU for 90 minutes to allow incorporation. BrDU positive cells were later detected using an Anti-BrDU staining kit from BD-pharmingen according to the manufacturer's instruction. Carboxyfluoroscein succinimidyl ester (CFSE) dye was purchased from Invitrogen to measure cell proliferation according to

manufacturer's instructions. Apoptotic cells were detected using an Annexin V staining kit from BD pharmingen.

Real-time PCR. Cell lysis was performed using Trizol reagent. The total RNA was reverse transcribed using the first strand cDNA synthesis kit from GE healthcare. Real-time PCR was performed using SYBR-green reagent from Applied Biosystems in ABI 7500 cyclers. Primers specific to the gene or region of interest were used for the levels of mRNA or CHIP assay respectively.

CLL transplantation. Whole splenocytes were isolated from mice with overt CLL. CLL was transplanted by intraperitoneal (IP) injections of 10^7 whole splenocytes into the sublethally irradiated (2 grays) NSG mice.

Statistical Analysis. Each experiment was repeated at least three times unless otherwise indicated. The data in the bar graphs are represented with \pm standard deviation. Two-tailed Student t-test was used to calculate p values to determine the significance. p value below 0.05 is considered statistically significant. Kaplan Meier survival analysis was performed using the log-rank test.

4.3 RESULTS

4.3.1 IRF4^{-/-}Vh11 CLL cells display hyperactive Notch signaling.

We hypothesized that Notch Signaling plays a critical role in the development of CLL in IRF4^{-/-}Vh11 mice. To test our hypothesis, we performed western blot analysis to measure the levels of canonical Notch target gene Hes1. Interestingly, Hes1 was significantly upregulated in IRF4^{-/-}Vh11 CLL cells compared to IRF4^{+/+}Vh11 B cells (Figure 1A). Notch protein family comprises of four different Notch paralogues from Notch1 through Notch4 in mammalian systems. We next wanted to identify the predominant Notch paralogue(s) expressed in the IRF4^{-/-}Vh11 CLL cells. Using

western blot analysis our studies revealed Notch2 protein as the predominant Notch paralogue expressed in IRF4^{-/-}Vh11 CLL cells (Figure 1B). The expression levels of Notch1 (Figure 1B), Notch3 and Notch4 (data not shown) were barely detectable or undetectable in IRF4^{-/-}Vh11 CLL cells. These findings are consistent with previous findings that described expression of Notch2 protein as the predominant Notch paralogue in mature murine B cells (28). Furthermore, the levels of Notch2 protein detected in IRF4^{-/-}Vh11 CLL cells were significantly higher compared to IRF4^{+/+}Vh11 B cells (Figure 1B). Additionally, we also used a flow cytometry based assay to measure the cell surface levels of Notch2 protein on IRF4^{-/-}Vh11 CLL cells. Consistent with western blot analysis, we identified a significant upregulation of Notch2 protein on cell surface of IRF4^{-/-} Vh11 CLL cells (Figure 1C). It is worth pointing out that we did not observe a significant change in the Notch2 mRNA expression suggesting that high levels of Notch2 protein in IRF4^{-/-}Vh11 CLL cells is likely a result of a post-transcriptional regulation. This conclusion is consistent with the results of a recent study describing an increase in Notch2 protein expression in IRF4 deficient mature B cells without corresponding changes in mRNA expression (22). Collectively, our results show hyperactivation of Notch signaling as a common feature of IRF4^{-/-}Vh11 CLL cells. Our results also specify Notch2 protein as the predominant Notch paralogue that is overexpressed in IRF4^{-/-} Vh11 CLL cells.

To confirm Notch2 as the predominant Notch receptor in B cells we tested the effect of *notch2* gene deletion on the intensity of Notch signaling *in vivo*. Notch2 deletion in B cells was achieved by breeding the CD19cre mouse to mouse carrying conditional alleles for *notch2* gene (CD19cre Notch2^{fl/fl}). Our result shows that the CD19cre Notch2^{fl/fl} mice underwent efficient Notch2 deletion leading to a significant decrease in Notch2 protein levels in mature B cells compared to B cells from CD19cre control mice (Figure 1D). The decrease in Notch2 protein was also accompanied by a dramatic downregulation of Notch target gene Hes1 in mature B cells, an indication of weakened Notch signaling *in vivo* (Figure 1E). The B cell development in CD19cre

Notch2^{fl/fl} mice was normal other than a defect in the generation of MZ B cell subset, as reported previously (28). Taken together, these results indicate that Notch2 protein is the major contributor of Notch signaling in mature B cells and its loss leads to a profound abrogation of Notch signaling *in vivo*. Moreover, the loss of Notch2 expression apparently is not compensated for by other Notch protein family members as indicated by a strong decrease in Hes1 levels in B cells from CD19cre Notch2^{fl/fl} mice.

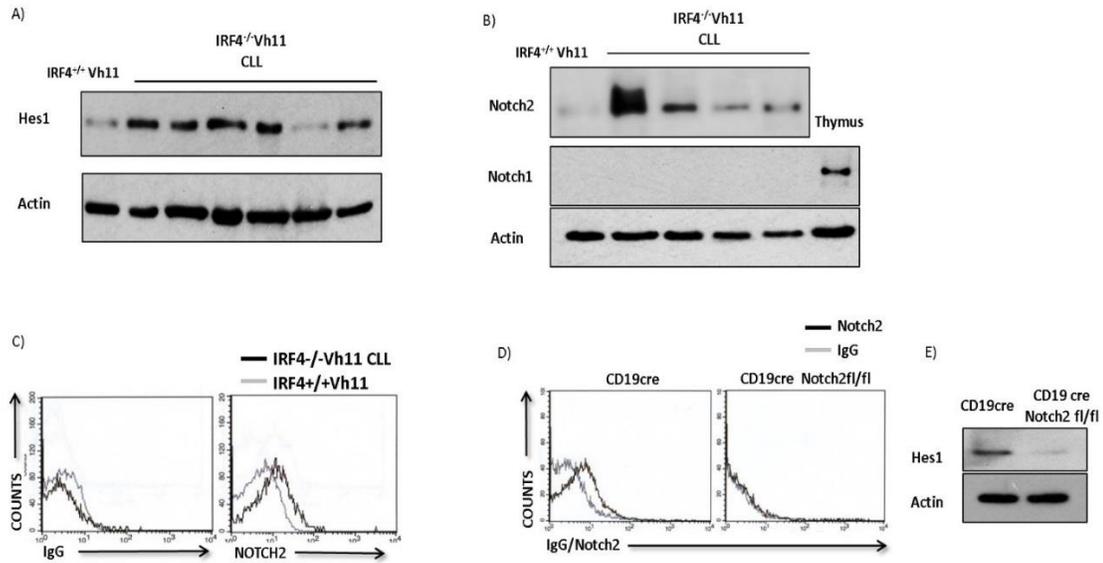


Figure 1 IRF4^{-/-}Vh11 CLL cells display hyperactive Notch signaling and express high levels of Notch2 receptor. (A) Western blot analysis to detect the levels of Hes1 protein in IRF4^{-/-}Vh11 CLL cells compared to IRF4^{+/+}Vh11 B cells isolated from spleen. Each lane represents CLL cells from an IRF4^{-/-}Vh11 mice. (B) Western blot to detect the levels of Notch2 and Notch1 proteins in IRF4^{-/-}Vh11 CLL cells. Each lane represents an individual CLL sample. Thymus is used as a positive control for Notch1 protein and actin is used as loading control. (C) Histograms showing Notch2 cell surface staining in IRF4^{-/-}Vh11 CLL cells compared to IRF4^{+/+}Vh11 B cells as detected by Flow cytometry. Left panel shows isotype control antibody (IgG) staining and right panel shows Notch2 staining. Gray line represents gating on IRF4^{+/+}Vh11 B cells and black line indicates IRF4^{-/-}Vh11 CLL cells. (D) Flow cytometry staining for IgG and Notch2 represented as histograms. Gray line represents IgG staining and black line represents Notch2 staining in B cells. Left panel indicates CD19cre control mouse and right panel shows CD19cre Notch2^{fl/fl} mouse. (E) Western blots showing Hes1 protein in B cells isolated from CD19cre and CD19cre Notch2^{fl/fl} mice. The data shown is representative of at least three independent experiments.

4.3.2 Notch signaling promotes the survival and proliferation of B1 cells and CLL cells.

We wanted to determine the effect of Notch signaling on B1 cells (CLL precursors) and CLL cells. To this end, we used an *in vitro* co-culture system to examine the impact of Notch signaling on their survival and proliferation. Briefly, we retro-virally transduced S17 stromal cells with a vector containing the Notch ligand, Delta like 1 (S17-DL1) to trigger Notch signaling. S17 cells transduced with the empty vector were used as controls (S17-R1). We first assessed the activation of Notch signaling by measuring the levels of Hes1 protein (Figure 2A). As expected, B1 cells cultured on S17-DL1 stromal cells showed strong activation of Notch signaling compared to cells cultured on S17-R1 stromal cells (Figure 2A). We then isolated B1 cells from the peritoneal cavities (PC) of CD19cre Notch2^{+/+} and CD19cre Notch2^{fl/fl} mice and cultured them on S17-R1 control or S17-DL1 Notch ligand expressing stromal cells. Interestingly, the CD19cre Notch2^{+/+} B1 cells cultured on S17-DL1 stromal cells proliferated significantly faster compared to the cells cultured on S17-R1 stromal cells as measured by BrdU incorporation assay (Figure 2B and 2C). This result indicates that Notch signaling promotes proliferation of B1 cells. Importantly, the increase in proliferation observed on wild type B1 cells was mostly abolished when Notch2 was deleted in B1 cells (CD19cre Notch2^{fl/fl}) (Figure 2B and 2C). Similarly, we also observed a decrease in apoptosis of CD19cre Notch2^{+/+} B1 cells, cultured on S17-DL1 stromal cells (Figure 2D and 2E). The increase in survival observed on S17-DL1 stromal cells was again negated in B1 cells isolated from CD19cre Notch2^{fl/fl} mice (Figure 2D and 2E).

We next examined the effect of Notch signaling on CLL cells derived from IRF4^{-/-}Vh11 mice. CFSE dilution assay revealed that IRF4^{-/-}Vh11 CLL cells cultured on S17-DL1 stromal cells proliferated much faster than their counterparts cultured on S17-R1 stromal cells (Figure 2F). Also, the survival of IRF4^{-/-}Vh11 CLL cells was enhanced when cultured on Notch ligand expressing (S17-DL1) stromal

cells (Figure 2G). In summary, these results demonstrate that CLL cells and their precursors are responsive to Notch signaling, which promotes their survival and proliferation.

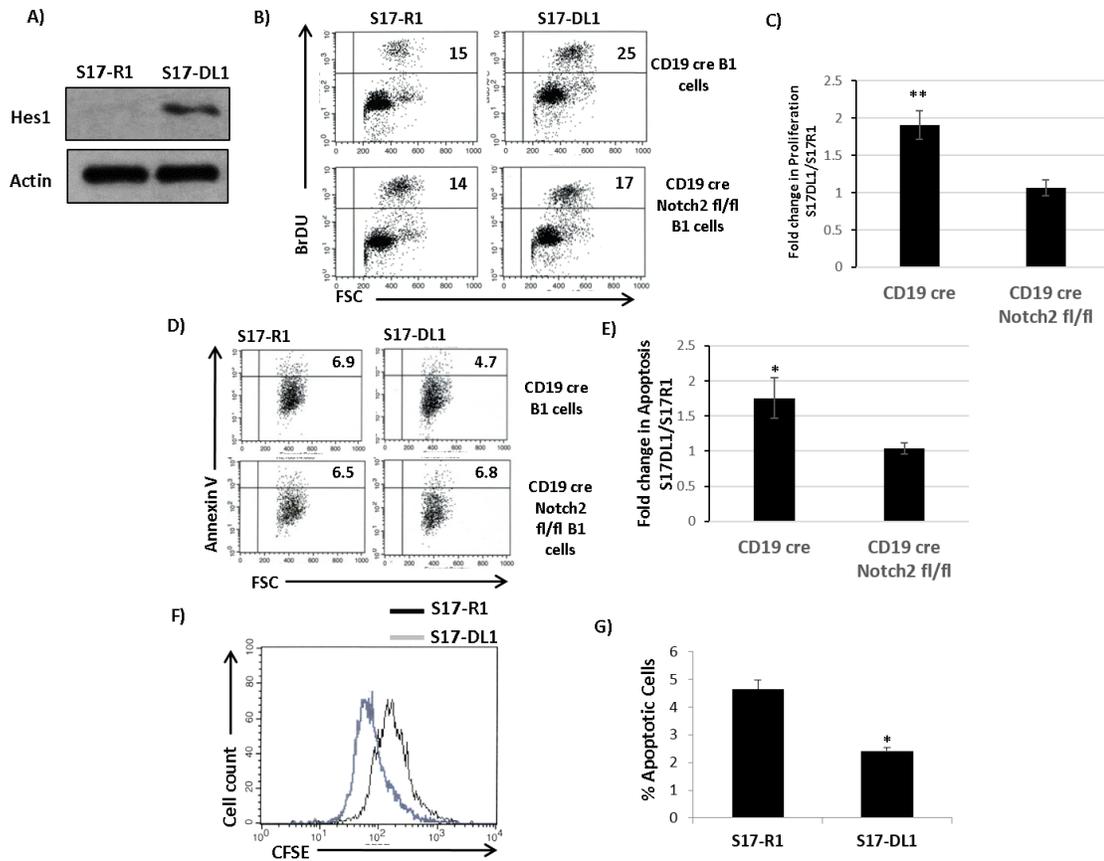


Figure 2 Notch signaling promotes the survival and proliferation of B1 cells and CLL cells. (A) Western Blot analysis to measure Hes1 levels in B1 cells co-cultured with S17-R1 and S17-DL-1 stromal cells for 48 hours. (B) Flow cytometry analysis showing the BrDU incorporation assay for cell proliferation of CD19cre control and CD19cre Notch2^{fl/fl} B1 cells co-cultured with S17-R1 and S17-DL1 stromal cells for 48 hours. The numbers in the upper right quadrant of each dot plot represents the percentage of BrDU positive cells. (C) Bar graphs showing the statistical analysis of BrDU incorporation assay from three independent experiments. The data is represented as fold change in proliferation observed on S17-DL1 stromal cells compared to S17-R1 control stromal cells. (D) Flow cytometry analysis showing Annexin V staining to detect apoptotic cells among CD19cre and CD19cre Notch2^{fl/fl} B1 cells cultured with S17-R1 and S17-DL-1 stromal cells for 48 hours. The numbers in each dot plot represents the percentage of Annexin V positive cells in the upper right quadrant. (E) Bar graph showing the statistical analysis of Annexin V staining of CD19cre and CD19cre Notch2^{fl/fl} B1 cells from five independent experiments. The data is represented as fold change in proliferation observed on S17-DL1 stromal cells compared to S17-R1 control stromal cells. (F) Histograms representing CFSE dye dilution experiment to measure proliferation of IRF4^{-/-}Vh11 CLL cells co-cultured with S17-R1 (black line) and S17-DL1 (gray line) stromal cells. Black line represents CLL cells cultured on S17-R1 stromal cells and gray line represents. (G) Bar graphs showing the percentages of Annexin V positive IRF4^{-/-}Vh11 CLL cells co-cultured with S17-R1 and S17-DL1 stromal cells from three independent experiments. *p value ≤ 0.01. **p value ≤ 0.05.

4.3.3 Notch2 in critical for CLL development in IRF4^{-/-} Vh11 mice.

We next wanted to determine the role of Notch signaling in the development of CLL in IRF4^{-/-}Vh11 mice. To address this goal we utilized a genetic approach to delete Notch2 gene in the IRF4^{-/-}Vh11 mice. Briefly, we bred the IRF4^{-/-}Vh11 mice with the CD19cre Notch2^{fl/fl} mice to generate CD19cre Notch2^{fl/fl} IRF4^{-/-}Vh11 mice (Notch2^{fl/fl} IRF4^{-/-}Vh11). Blood was analyzed biweekly from Notch2^{fl/fl} IRF4^{-/-}Vh11 mice to monitor the emergence of CLL cells and CD19cre IRF4^{-/-}Vh11 mice were also analyzed as control. Interestingly, compared to CD19cre IRF4^{-/-}Vh11 mice (n=18) we observed a significant delay in the onset of CLL development in CD19cre Notch2^{fl/fl} IRF4^{-/-}Vh11 mice (n=11) (Figure 3A). The disease latency increased from 19.5 weeks in CD19cre IRF4^{-/-}Vh11 mice to 28.8 weeks in Notch2^{fl/fl} IRF4^{-/-}Vh11 mice (Figure 3A). Surprisingly, upon further analysis we observed that the CLL cells which emerged from Notch2^{fl/fl} IRF4^{-/-}Vh11 mice, continued to express high levels of Notch2 protein on their cell surface (Figure 3B right panel). In total, we analyzed 15 mice with Notch2^{fl/fl} IRF4^{-/-}Vh11 genotype and all of them eventually showed emergence of CLL cells which retained Notch2 expression on their cell surface. These results can have three plausible explanations. Firstly, these findings can be caused by insufficient CD19cre mediated Notch2 deletion in B cells of the IRF4^{-/-}Vh11 mice. Secondly, these findings can also be caused by a defect in B cell development upon Notch2 deletion that prevents the generation of CLL precursors (B1 cells) in the IRF4^{-/-}Vh11 mice. Thirdly, these findings can be explained by our hypothesis which implies that Notch2 is critical for CLL development and without it, CLL cells cannot be generated.

To distinguish between these different scenarios, we analyzed Notch2 expression in Notch2^{fl/fl} IRF4^{-/-}Vh11 mice that were still at the early stages of CLL development. This allowed us to simultaneously evaluate a CLL population as well as a detectable population of untransformed normal B cells (B2 cells) in the same mice. Intriguingly, our analysis revealed that only the CLL cells from Notch2^{fl/fl} IRF4^{-/-}Vh11 mice expressed high levels of Notch2 protein while, the normal B cells

from the same mice displayed very low to undetectable levels of Notch2 protein (Figure 3B right panel). These findings were consistent in cells isolated from several tissue including peritoneal cavity (PC), spleen and blood (Figure 3B). Concurrently, we devised a real-time PCR based assay to precisely calculate the efficiency of Notch2 deletion among different cell populations. We specifically designed PCR primers in region within the Notch2 conditional allele that is flanked by the loxP sites. This approach allows for PCR amplification from Notch2 alleles that have not undergone cre mediated deletion. Furthermore, we also amplified a non-related region in the genome and used it as control to normalize the result. This method precisely calculates absolute deletion efficiencies for the *notch2* alleles. To validate this method, we extracted genomic DNA from B cells of wildtype B6 and CD19cre Notch2^{fl/+} mice. As expected, the assay revealed a Notch2 deletion efficiency of 47% in B cells isolated from CD19cre Notch2^{fl/+} heterozygous mice compared to wildtype B cells (Figure 3C). Using this assay we first wanted to rule out the possibility for any aberrant B cell developmental defect in Notch2^{fl/fl} IRF4^{-/-}Vh11 mice. To this end, we analyzed Notch2^{fl/fl} IRF4^{-/-}Vh11 mice of 2-3 months of age with no overt signs of CLL. Flow cytometry analysis showed efficient generation of CD5+IgM+ B1 cells in the peritoneal cavities of Notch2^{fl/fl} IRF4^{-/-}Vh11 mice at a frequency that is comparable to that of IRF4^{+/+}Vh11 and IRF4^{-/-}Vh11 mice (Figure 3D). Furthermore, the B1 and B2 (normal B cells) cells isolated from Notch2^{fl/fl} IRF4^{-/-}Vh11 mice with no CLL displayed very high efficiencies of *notch2* gene deletion (~90%) (Figure 3C Box1). These results indicate that Notch2 is not essential for B1 cell generation in the Notch2^{fl/fl} IRF4^{-/-}Vh11 mice. Together, our results rule out the first explanation by demonstrating that *notch2* gene is efficiently deleted in all B cell subsets in the Notch2^{fl/fl} IRF4^{-/-}Vh11 mice. Additionally, our results also discredit the second explanation by showing that Notch2 is dispensable for the generation of B1 cells in the IRF4^{-/-}Vh11 mice.

We next used FACS to sort CLL cells and normal B cells (B2 cells) from *Notch2^{fl/fl} IRF4^{-/-}Vh11* mice and extracted genomic DNAs from sorted cells for analyzing the respective *notch2* deletion efficiencies. The normal B cells (B2 cells) isolated from *Notch2^{fl/fl} IRF4^{-/-}Vh11* displayed high efficiency of *notch2* deletion ($\geq 90\%$) (Figure 3C Box2). Whereas, the CLL cells from the same mice displayed significantly lower *notch2* gene deletion efficiencies (Figure 3C Box2). It appears that CLL cells in some mice (mice 1 and 2) completely escaped *notch2* gene deletion (close to 0% deletion efficiency) while, in other mice (mice 3 and 4) the CLL cells showed 30-40% *notch2* gene deletion efficiencies (Figure 3C Box2). 30-40% deletion efficiency in these mice may reflect a mixed CLL population with heterozygous *Notch2* deletion. However, it is worth pointing out that even in those mice, we did not observe a corresponding decrease in the *Notch2* protein levels in the CLL populations (data not shown). In summary, our studies here show that *Notch2* is indispensable for the generation of CLL in *IRF4^{-/-}Vh11* mice, indicating that Notch signaling is critical for CLL development in *IRF4^{-/-}Vh11* mice.

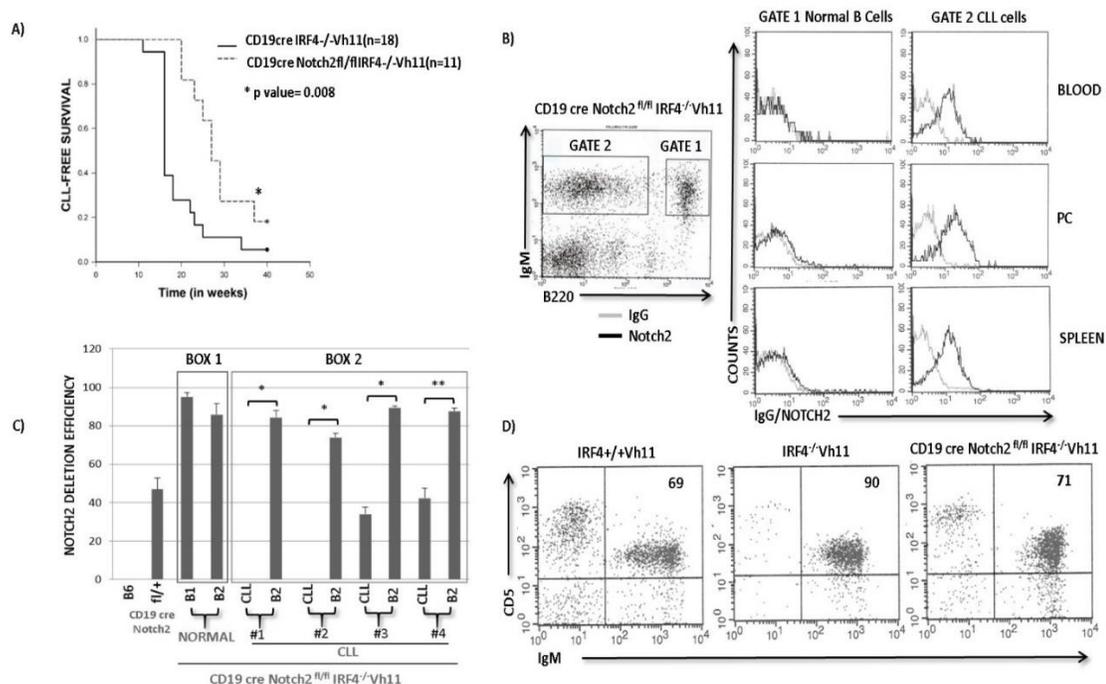


Figure 3 **Notch2 receptor is critical for CLL development in IRF4^{-/-}Vh11 mice.** (A) Kaplan Meier Survival analysis (log-rank test) for CLL development in CD19cre Notch2^{fl/fl} IRF4^{-/-}Vh11 mice (n=11) (dashed line) compared to CD19cre IRF4^{-/-}Vh11 mice (n=18) (solid line). Blood was analyzed biweekly to monitor CLL development that is considered as an event represented on Y-axis. X-axis represents time in weeks. (B) Left panel shows flow cytometry staining of IgM and B220 in CD19cre Notch2^{fl/fl} IRF4^{-/-}Vh11 mice. Normal untransformed B cells are IgM⁺ and B220 high (Gate 1) and CLL cells are IgM⁺ and B220 medium/dim (Gate 2). Right panel shows histograms representing IgG (gray line) or Notch2 (black line) staining in Normal B cells and CLL cells from Blood, Peritoneal Cavity (PC) and Spleen. (C) Bar graph showing qRT-PCR data representing absolute Notch2 deletion efficiencies. A deletion efficiency of 47 as observed in CD19cre Notch2^{fl/fl} B cells signifies 47% *notch2* gene deletion. Box1 contains B1 (CLL precursors) and B2 (normal B) cells from CD19cre Notch2^{fl/fl} IRF4^{-/-}Vh11 mice without overt signs of CLL. Box2 encloses Notch2 deletion efficiencies in CLL and B2 cells from four different CD19cre Notch2^{fl/fl} IRF4^{-/-}Vh11 mice with overt CLL. (D) Flow cytometry staining to detect IgM⁺ CD5⁺ B1 cells in peritoneal cavities of IRF4^{+/+}Vh11, IRF4^{-/-}Vh11 and CD19cre Notch2^{fl/fl} IRF4^{-/-}Vh11 (no CLL) mice. The numbers represents the frequency of B1 cells. *p value ≤0.001 **p value ≤0.01.

4.3.4 Notch2 is important for maintenance of CLL cells in IRF4^{-/-}Vh11 mice.

We next generated an inducible Notch2 deletion model to study the role of Notch Signaling in the maintenance of CLL cells in IRF4^{-/-}Vh11 mice. To accomplish this, we used a genetic approach to delete *Notch2* gene in IRF4^{-/-}Vh11 CLL cells. We bred the IRF4^{-/-}Vh11 mice with the ERTcreNotch2^{fl/fl} to eventually generate ERTcreNotch2^{fl/fl}IRF4^{-/-}Vh11 mice (Figure 4A). The blood of ERTcreNotch2^{fl/fl}IRF4^{-/-}Vh11 mice was analyzed biweekly to monitor overt CLL development. Upon CLL development, the CLL cells from ERTcreNotch2^{fl/fl}IRF4^{-/-}Vh11 were transplanted to NOD-scid Gamma chain deficient (NSG) immunodeficient mice (Figure 4A). The transplanted NSG mice were then injected either with tamoxifen to activate cre recombinase or with corn oil (Figure 4A). After tamoxifen injections, blood was analyzed biweekly to monitor Notch2 protein expression. As expected, tamoxifen treatment led to a decrease in the cell surface expression of Notch2 protein on CLL cell population by 3weeks (Figure 4B). Interestingly, the initial decrease was followed by restoration of Notch2 cell surface levels on CLL cells from NSG mice treated with tamoxifen by 5 weeks (Figure 4B). Concomitantly, we calculated the absolute *Notch2* deletion efficiencies in tamoxifen treated and control NSG mice. Our results revealed a strong correlation between Notch2 cell surface expression and *Notch2* deletion efficiencies in NSG mice treated with tamoxifen over the course of time (Figure 4C). The CLL cells from NSG mice treated with tamoxifen showed ~ 70% *Notch2* deletion by week 3 which decreased to ~10% by week 5 (Figure 4C). However, only a few mice were used for these studies and further studies with a larger cohort of mice are needed to confirm these results. In summary, these studies indicate an important role Notch2 receptor and the associated Notch signaling in the maintenance of CLL cells in IRF4^{-/-}Vh11 mice.

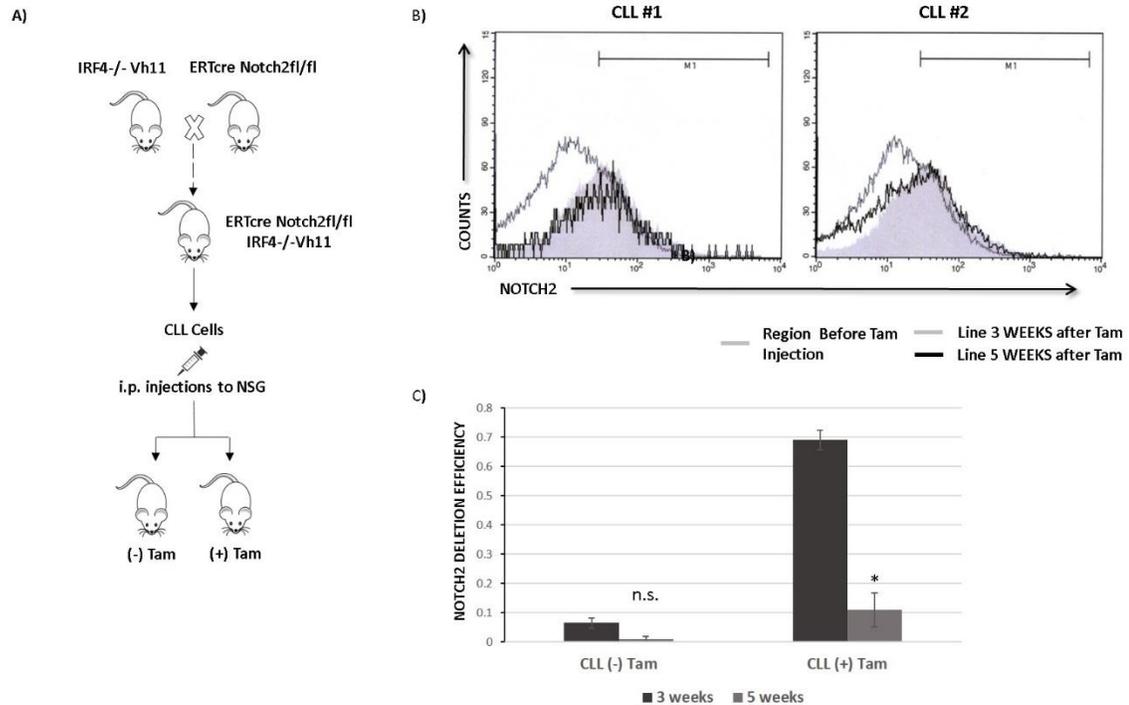


Figure 4 **Notch2 receptor is important for CLL maintenance in IRF4^{-/-}Vh11 mice.** (A) A schematic showing the generation of ERTcreNotch2^{fl/fl}IRF4^{-/-}Vh11 mice and the experimental design to induce Notch2 deletion upon tamoxifen treatment. (B) Histograms showing the Notch2 cell surface expression of CLL cells isolated from NSG mice transplanted with ERTcreNotch2^{fl/fl}IRF4^{-/-}Vh11 CLL cells and treated with tamoxifen. Gray region in each panel indicates Notch2 cell surface before tamoxifen injections. Gray line and black line represents Notch2 expression 3weeks and 5 weeks post tamoxifen treatment respectively. Each panel represents cells CLL cells isolated from a different NSG mouse treated with tamoxifen(C) Bar graph showing qRT-PCR data representing absolute Notch2 deletion efficiencies. Gray bars represent Notch2 deletion efficiencies at 3 weeks and black bars represents deletion efficiency after 5 weeks. *p value ≤ 0.005 .

4.4 DISCUSSION

Genetic evidence point towards an important role for Notch signaling in the pathogenesis of CLL (2, 5). However, the significance of Notch signaling in the development of CLL *in vivo* has not been examined. Our studies here provide the first *in vivo* genetic evidence that Notch signaling is essential for development of CLL. IRF4^{-/-}Vh11 mouse is a novel model to study the pathogenesis of CLL (18, 23). Our results show that similar to many human CLL cases, Notch signaling is hyperactive in IRF4^{-/-}Vh11 CLL cells. To examine the importance of Notch signaling in CLL development, we used a genetic approach to delete Notch2 gene in IRF4^{-/-}Vh11 mice. Our studies reveal an absolute requirement of *notch2* gene for the generation of CLL cells, as CLL cells which eventually emerged from Notch2^{fl/fl} IRF4^{-/-}Vh11 mice were always the ones that escaped cre-mediated Notch2 deletion. Importantly, our findings are not caused by defects in B1 cell development, as B1 cells can still be generated in the Notch2^{fl/fl} IRF4^{-/-}Vh11 mice at relatively normal frequency.

Our findings support a role of Notch signaling in CLL initiation. This conclusion is supported by our results showing a significant delay in the onset of CLL upon *notch2* gene deletion. The Notch2^{fl/fl} IRF4^{-/-}Vh11 mice showed a significant increase in disease latency compared to CD19cre IRF4^{-/-}Vh11 mice. Moreover, our results show that Notch signaling promoted the survival and proliferation of CLL precursors (B1 cells) which may directly contribute to CLL initiation *in vivo*. A role for Notch in CLL initiation is further supported by a recent genomic analysis which shows that Notch mutations can be detected in early hematopoietic progenitor cells of CLL patients (29). The frequency of Notch mutations are dramatically increased in therapy-resistant CLL patients, indicating a role of Notch in disease progression (1, 2). Intriguingly, we also observed a detrimental effect on CLL cells survival and proliferation when *notch2* gene was deleted in the

IRF4^{-/-}Vh11 mice after onset of CLL with an inducible cre. This result would indicate that Notch signaling is also important for CLL maintenance.

The survival and proliferation of CLL cells have been shown to depend on diverse signaling cues emanating from their specialized tumor microenvironment (30). Notch signaling presumably plays a role in these specialized microenvironmental sites to promote the survival and proliferation of CLL cells (10, 31). Our previous studies identified spleen as the primary site of proliferation for CLL cells in IRF4^{-/-}Vh11 mice (18). Additionally, recent studies using patient derived xenograft models have also identified murine spleen as the primary site for the homing, survival and proliferation of human CLL cells (32). Intriguingly, murine spleen has been described as a specialized anatomical location expressing high levels of Notch ligands (33, 34). It is likely that the high concentrations of Notch ligands and subsequent activation of Notch signaling may contribute greatly to the enhanced survival and proliferation of IRF4^{-/-}Vh11 CLL cells in the murine spleen.

In summary, we show that Notch2 receptor and the associated signaling is upregulated in CLL cells from IRF4^{-/-}Vh11 mice. We show that Notch signaling promotes the survival and proliferation of CLL cells and their precursors. We describe IRF4^{-/-}Vh11 mice as a novel model system to study the role of Notch signaling in the pathogenesis of CLL. Our studies presented here uncover an important role of Notch signaling in the development of CLL in IRF4^{-/-}Vh11 mice. However, the molecular mechanism that leads to deregulation of Notch signaling in IRF4^{-/-}Vh11 mice was still unclear.

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CHAPTER 5

IRF4 attenuates Notch signaling by regulating the E3 ubiquitin Ligase Nedd4

5.1 INTRODUCTION

Recent Whole Genome Sequencing (WGS) studies have provided valuable insights into the molecular pathways deregulated during the pathogenesis of Chronic Lymphocytic Leukemia (CLL). Intriguingly, multiple WGS studies have identified mutational activation of Notch signaling pathway as one of the most recurrent molecular event in human CLL (1-5). Moreover, the CLL patients carrying mutations in Notch signaling pathway have poor clinical outcomes and an increased tendency towards Richter transformation to Diffused Large B cell Lymphoma (DLBCL) (1, 2, 6). Notch signaling is an evolutionarily conserved pathway that regulates a myriad of cellular processes (7). Notch signaling is activated upon engagement of Notch receptor on a signal receiving cell with its ligand on a signal sending cell (7). Following the ligand binding Notch receptor undergoes multiple proteolytic cleavages leading to translocation of intracellular domain of Notch to the nucleus (7). In the nucleus Notch intracellular domain functions as a transcriptional regulator to activate gene transcription of its target genes (7). In CLL patients, Notch signaling pathway can be activated by mutations that primarily affect the stability of Notch1 protein (2, 5). Notch mutations in CLL patients cause frameshift deletions leading to generation of protein without the PEST domain (2, 5). The PEST domain in Notch proteins are functionally important for ubiquitination and degradation of Notch proteins and hence for limiting the cellular responses to Notch signaling (7). Other than the mutational activation, studies have also reported constitutively high expression of Notch1 and Notch2 leading to activation of Notch signaling in human CLL cells (8). *In vitro* studies relying on co-culture of CLL cells with stromal cells have provided evidence for a role of Notch signaling in promoting the survival and chemo-resistance of CLL cells (9, 10). Although, these studies have linked aberrant Notch signaling to the pathogenesis of CLL *in vitro*, whether Notch signaling is critical for CLL development *in vivo* remains unknown.

Furthermore, the molecular pathways that lead to the deregulated Notch signaling in CLL cases without Notch mutations are still poorly defined.

Interestingly, a recent study described expansion of a specialized mature B cell subset known as Marginal Zone B cells (MZ B cells) in IRF4 deficient mice (11). Moreover, the expansion of MZ B cells were attributed to higher levels of Notch2 receptor and associated Notch signaling in the IRF4 deficient mature B cells (11). Although the precise mechanism through which IRF4 regulates Notch signaling remains unclear, this study identified IRF4 as a potential novel regulator of Notch signaling in mature B cells. Given the possible connection between Notch signaling and CLL development, we hypothesized that in the IRF4^{-/-}Vh11 mice Notch signaling is also deregulated and the deregulation plays a critical role in CLL development. IRF4^{-/-}Vh11 mouse is regarded as a novel mouse CLL model because it mimics a predominant genetic predisposition to CLL (12). We used the IRF4^{-/-}Vh11 mice in our previous studies to identify a critical role for Notch signaling in the development of CLL (Chapter 4). These studies provide the first *in vivo* evidence for an important role of Notch signaling in the development of CLL (Chapter 4). In the present studies we examined the molecular mechanism for regulation of Notch signaling by IRF4 during the development of CLL in IRF4^{-/-}Vh11 mice.

5.2 MATERIALS AND METHODS

Animal Studies. IRF4^{-/-}Vh11 mice were generated and monitored for CLL development as previously described (13). Notch2 floxed (14), Rosa-rtTA (15) and CD19cre (16) mice were generated as described previously and purchased from Jackson laboratory. NOD-scid gamma chain deficient mice were obtained from Jackson laboratory. TRE IRF4 transgenic mice were generated and treated with doxycycline as previously described (17). All experiments were performed according to the guidelines from National Institute of Health and with an approved

protocol from Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Human Studies. All the human samples were collected and processed according to an approved protocol from Institutional Review Board. An informed written consent was obtained from each participant. The cells were isolated as previously described (18).

TABLE T1

CLL Patient #	Source	Cytogenetics	IgVH mutation status	CD38 positivity	Monotypic B-cells
CLL 1	PB	Trisomy 12	Unmutated	Pos	93%
CLL 2	PB	11q23, 13q14 del	Unmutated	Neg	95%
CLL 3	PB	11q23	Not done	Pos	74%
CLL 4	PB	17p del, 13q	Unmutated	Pos	92%
CLL 5	PB	Normal	Mutated	Neg	82%
CLL 6	PB	13q14 del	Unmutated	Neg	61%
CLL 7	PB	13q14 del	Not done	Neg	53%
CLL 8	PB	13q14 del	Not done	Neg	74%
CLL 9	PB	Normal	Mutated	Pos	78%
CLL 10	PB	13q null	Not done	Neg	75%
CLL 11	PB	Normal	Not done	Pos	43%
CLL 12	PB	11q23, 13q14 del	Not done	Neg	93%

CLL 13	PB	Normal	Not done	Pos	85%
CLL 14	PB	Trisomy 12	Not done	Neg	77%
CLL 15	PB	Normal	Mutated	Neg	97%
CLL 16	PB	13q14 del	Not Done	Neg	80%
CLL 17	PB	13q14 del	Not Done	Neg	85%
CLL 18	PB	11q23, 13q14 del	Unmutated	Neg	67%

Chromatin Immuno-precipitation (ChIP) Sequencing. B1 cells were isolated and fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by the addition of lysis buffer. Lysates were sonicated and the DNA sheared to an average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. An aliquot of chromatin (30 ug) was precleared with protein G agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using 4 ug of antibody against IRF4 (Santa Cruz, sc-6059). Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation.

Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on Illumina's HiSeq 2500 (50 nt reads, single end). Reads were aligned to the mouse genome (mm10) using the BWA algorithm (default settings). Duplicate reads were removed and only uniquely mapped reads

(mapping quality ≥ 25) were used for further analysis. Alignments were extended in silico at their 3'-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The resulting histograms (genomic "signal maps") were stored in bigWig files. Peak locations were determined using the MACS algorithm (v1.4.2) with a cutoff of p-value = $1e-7$. Signal maps and peak locations were used as input data to Active Motifs proprietary analysis program (19), which creates Excel tables containing detailed information on sample comparison, peak metrics, peak locations and gene annotations. The representative data is generated by analyzing the data using the Integrated Genome Browser (IGB).

RNA preparation and next-generation sequencing: Total RNA was extracted from IRF4-/-Vh11 CLL cells with or without IRF4 induction using Trizol Reagent, and submitted to Otogenetics Corporation (Norcross, GA USA) for RNA-Seq assays. Briefly, the integrity and purity of total RNA were assessed using Agilent Bioanalyzer or Tapestation and OD260/280. 1-2 μ g of cDNA was generated from high quality total RNA using the Clontech SMARTer cDNA kit, polyA primer, (Clontech Laboratories, Inc., Mountain View, CA USA, catalog# 634926). The resulting cDNA was fragmented using Bioruptor (Diagenode, Inc., Denville, NJ USA), profiled using Agilent Bioanalyzer or Tapestation. Illumina libraries were made from qualified fragmented gDNA using SPRIworks HT Reagent Kit (Beckman Coulter, Inc. Indianapolis, IN USA, catalog# B06938) on the Biomek FXp liquid handler (robot). The quality and quantity and the size distribution of the Illumina libraries were determined using an Agilent Bioanalyzer or Tapestation. The libraries were then sequenced on Illumina HiSeq2500, using RapidRun V1 chemistry, paired-end 106 nucleotide reads, according to the standard operation. Reads were generated and checked for data quality using FASTQC (Babraham Institute, Cambridge, UK).

The data sets was mapped against reference genome mm10 with star 2.4.0j, and then analyzed with cufflinks (2.2.1) for expression level measurement on www.DNAnexus.com. Comparison of expression level between samples had been conducted with cufflinks.cuffdiff (2.2.1).

CLL transplantation. Whole splenocytes were isolated from mice with overt CLL. CLL was transplanted by intraperitoneal (IP) injections of 10^7 whole splenocytes into the sublethally irradiated (2 grays) NSG mice.

Western Blotting. B cells and CLL cells from spleen were isolated by negative selection using MACS columns. Lysates were prepared and resolved using SDS-PAGE. The membranes were incubated with the indicated antibodies and Horse radish peroxidase (HRP) conjugated secondary antibodies. The signals were generated using Enhanced Chemi-Luminescence (ECL) substrate solution from Thermo-Pierce. The antibodies against Notch1, Notch2, Itch, Nedd4 and Hes1 were purchased from Cell signaling Technologies. Antibodies against IRF4 and Pu.1 were obtained from Santa Cruz Biotechnology. Direct HRP conjugated antibody against Beta Actin was purchased from Sigma.

Transfection of Mec-1 CLL and normal B cells. Mec-1 cells were transfected using the Solution V kit purchased from Lonza. The transfection were carried out in a Nucleofector (Lonza) using the program X-001. Normal human B cells were isolated from the peripheral blood of healthy donors using MACS magnetic beads separation. Transfections of normal human B cells were carried out in a Nucleofector (normal human B cell solution) using the program U-015. The siRNA against human IRF4 (on-target plus smart pool) were purchased from Dharmacon (L-019668-00-0005). The siRNA against human Nedd4 (on-target plus smart pool) were purchased from Dharmacon (L-007178-00-0005). The ON-target plus Non targeting siRNA purchased from Dharmacon were used as controls (D001810-10-05). The cells were analyzed 48 hours post transfections.

Flow cytometry and cell sorting. The cells were isolated from respective tissues and pre-incubated with Fc-Block antibody. Flow cytometry staining was performed by incubation of cells with optimal amounts of biotinylated or directly fluorophore conjugated antibodies. Antibodies against mouse B220, IgM and CD5 proteins were purchased from BD-pharmingen. Anti-mouse Notch2 and the corresponding isotype control antibodies were purchased from Biolegend. The anti-IRF4 antibody and the corresponding control antibody for intracellular staining were purchased from ebioscience. Fluorescence activated cell sorter (FACS) analysis was performed using FACSCalibur flow cytometer. Cell sorting was performed using BD FACSAria flow cytometer.

Primary and Cell Cultures. All primary cells and Mec-1 CLL cell line were cultured in RPMI-1640 media containing 10% fetal bovine serum, 50 μ M Beta mercaptoethanol, 2mM L-glutamine and 100 U of penicillin and streptomycin. The B1 cells were isolated from peritoneal ascites following incubation in the tissue culture dishes for 6 hours to remove adherent macrophages.

Real-time PCR. Cell lysis was performed using Trizol reagent. The total RNA was reverse transcribed using the first strand cDNA synthesis kit from GE healthcare. Real-time PCR was performed using SYBR-green reagent from Applied Biosystems in ABI 7500 cyclers. Primers specific to the gene or region of interest were used for the levels of mRNA or CHIP assay respectively. A complete list of primers is included in the table 2 (Table T2).

Table T2

Primer	Forward	Reverse
Notch2 Deletion	ATGTCCAGAGGGCTTCTTGGGAG	TGGCAGTCCTCTCTGTGAATCC
Control Deletion	CTTCCTTTAGGGTAACTGGCCGCC	CAGGATAAAGGACACTCCACCCAG
Kappa CHIP	TAGCACAGAGTACCCACCCATATCTC	CTATCTTGGTCCATGGGACACTCCC
Nedd4 CHIP 2Kb	TGTTTCGGCTCATAATCTCATGGG	AACTGCACTACCACACCTGGCA

Nedd4 CHIP 4kb	CTGCCAGTGAAGCAGGAGCCTTTAG	GGGACCTAGAGTGGGCATCAAAA
human Nedd4 mRNA	ATGGCAACATTCAACTGCAA	GGCCTGGTTGCTATACATGG
human Fbxw7 mRNA	GACGCCGAATTACATCTGTC	GTAGCAGGTCTTTGGGTTTC
human Hes1 mRNA	AGTGAAGCACCTCCGGAAC	TCACCTCGTTCATGCACTC
mouse Hes1 mRNA	GTGTCAACACGACACCGGACAAACC	GCTTGAATGCCGGGAGCTATCT
mouse Notch2 mRNA	CAGCTCTAACCCGTGCCTGAATG	GGGCCTTGCCTTTTCTGAACAC
mouse Nedd4 mRNA	TCACTGCTGATCCGTACCTGGAGC	GCTGGTAAGGATTCCACTCATCGGG
mouse Fbxw7 mRNA	TGCAAAGTCTCAGATTATACC	ACTTCTCTGGTCCGCTCCAGC

Statistical Analysis. Each experiment was repeated at least three times unless otherwise indicated. The data in the bar graphs are represented with \pm standard deviation. Two-tailed Student t-test was used to calculate p values to determine the significance. p value below 0.05 is considered statistically significant.

5.3 RESULTS

5.3.1 IRF4 regulates the E3 ubiquitin ligase Nedd4 in IRF4^{-/-}Vh11 CLL cells.

Our results show that IRF4^{-/-}Vh11 CLL cells express high levels of Notch2. A previous study also shows that Notch2 expression levels are high in IRF4 deficient B2 cells (11). However, how the expression levels of Notch2 are regulated by IRF4 remains unclear. In an attempt to decipher the molecular mechanism, we reconstituted the expression of IRF4 in CLL cells. To accomplish this, we used a previously described IRF4 transgenic line where the expression of IRF4 transgene is driven by a tetracycline response element (TRE-IRF4) (17). A transgenic mouse expressing a reverse tetracycline-controlled transactivator (rtTA) was used to drive the expression of IRF4 transgene *in vivo*. The rtTA transgene along with an EGFP reporter were inserted in mouse Rosa

26 locus and can only be activated by cre recombinase (15). We bred mice containing rtTA and TRE IRF4 alleles to CD19cre mice to generate CD19cre rtTA TRE IRF4 mice which upon treatment with doxycycline (Dox), allow IRF4 transgene expression exclusively in B cells. Through further breeding we introduced these alleles (CD19cre rtTA TRE IRF4) into the IRF4^{-/-}Vh11 mice to generate CD19cre rtTA TRE IRF4 IRF4^{-/-}Vh11 mice hereafter referred to as IRF4^{-/-}Vh11Tg in this manuscript (Figure 1A). The blood was analyzed regularly from IRF4^{-/-}Vh11Tg mice. Upon CLL development in IRF4^{-/-}Vh11Tg mice, the CLL cells were isolated from spleen and transplanted to NOD-scid gamma deficient (NSG) immunocompromised mice (Figure 1A). The blood from the NSG mice was screened for the development of overt CLL. Upon successful establishment of CLL in the NSG mice, some mice were fed with dox containing water to induce the expression of IRF4 (NSG (+) Dox) (Figure 1A). NSG mice fed with regular water without dox were used as controls (NSG (-) Dox). This approach allowed us to compare the effect of IRF4 on the same population of CLL cells.

After three weeks of Dox treatment, we examined the effect of IRF4 reconstitution on the levels of Notch2 expression in IRF4^{-/-}Vh11Tg CLL cells. Interestingly, compared to NSG control mice IRF4 reconstitution led to a decrease in the cell surface levels of Notch2 receptor on CLL cells (Figure 1B). This is evidenced by a leftward shift in Notch2 staining of CLL cells in dox treated NSG mice in both blood and spleen (Figure 1B). We further performed a western blot analysis to measure the total levels of Notch2 protein in IRF4^{-/-}Vh11Tg CLL cells. Consistent with the result of FACS staining, we observed a decrease in total levels of Notch2 protein in CLL cells upon IRF4 induction (Figure 1C). Furthermore, we did not observe a corresponding decrease in the mRNA expression of Notch2 upon IRF4 induction (Figure 1D). These results indicate that IRF4 downregulates expression of Notch2 and that the defect in Notch2 expression can be corrected upon IRF4 reconstitution in IRF4^{-/-}Vh11 mice.

We further performed RNA sequencing (RNA-seq) from CLL cells isolated from mice treated with or without Dox. Our objective was to identify differentially expressed genes that could affect Notch protein turnover. A previous study has linked reduced expression of an E3 ubiquitin ligase gene *Fbxw7* to the increased Notch protein levels in the IRF4 deficient B cells (11). However, *Fbxw7* expression was not significantly affected upon IRF4 reconstitution (Figure 1F). Intriguingly, our RNA-seq data revealed an increase in expression of a different E3 ubiquitin ligase, *Nedd4* upon IRF4 reconstitution (Figure 1E). Importantly, *Nedd4* has been previously shown to ubiquitinate and degrade Notch receptors in drosophila and mammalian cellular systems (20-23). We were able to confirm RNA-seq results by real-time PCR (Figure 1F). Notably, reconstitution of IRF4 also led to a decrease in the expression of canonical Notch target gene *Hes1* (Figure 1E and 1F). Western blot analysis further showed a profound increase in *Nedd4* protein expression upon IRF4 reconstitution whereas the levels of another E3 ubiquitin ligase, *Itch* which belongs to the same protein family as *Nedd4*, remained unchanged (Figure 1G). We further analyzed the mRNA and protein expression of *Nedd4* in CLL cells. Compared to IRF4^{+/+}Vh11 B cells, the mRNA and protein levels of *Nedd4* were dramatically reduced in IRF4^{-/-}Vh11 CLL cells (Figure 1H and 1I). Taken together, these studies identify *Nedd4* as a potential IRF4 target gene and the major E3 ubiquitin ligase that is downregulated in the IRF4^{-/-}Vh11 CLL cells.

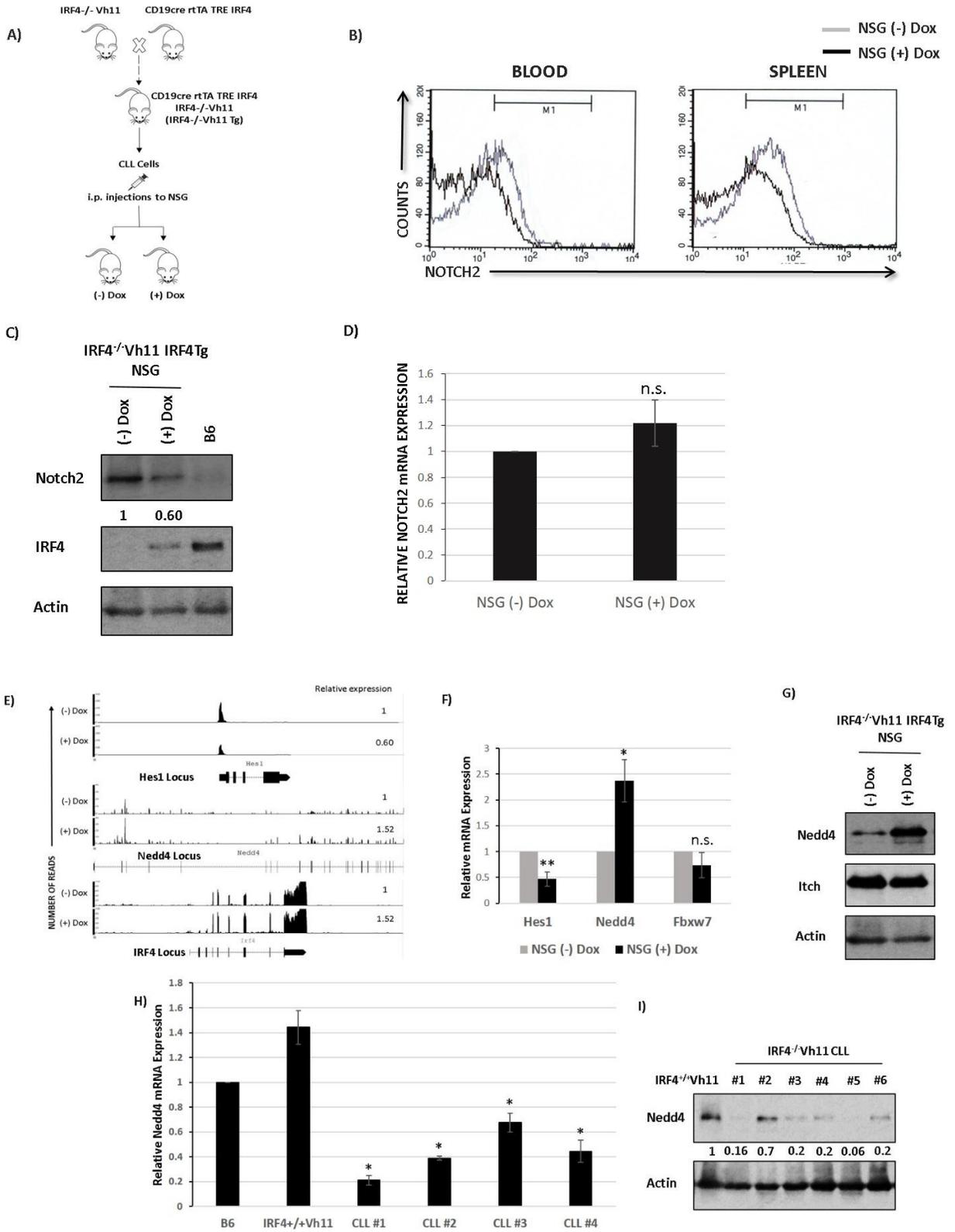


Figure 1 IRF4 regulates E3 ubiquitin ligase Nedd4 in CLL cells. (A) A schematic showing the breeding scheme and experimental design for IRF4 reconstitution in IRF4^{-/-}Vh11 CLL cells. (B) Histograms showing Notch2 staining in CLL cells isolated from blood and spleen of NSG mice fed with (black line) or without dox water for 3 weeks (gray line). (C) Western blot analysis to detect Notch and IRF4 levels in CLL cells isolated from NSG mice fed with or without dox water for 3 weeks. The number below represents normalized relative expression. B cells isolated from B6 mice are used as a measure of endogenous levels of IRF4. Actin is used as loading control. (D) Bar graph showing relative mRNA expression of Notch2 in CLL cells isolated from NSG mice fed with or without dox. (E) RNA sequencing data of CLL cells isolated from NSG mice fed with or without dox water. Each insert shows representative data for Hes1, Nedd4 and IRF4 genes. The numbers on the right corner represents relative expression in CLL cells from respective NSG mice. (F) Bar graph representing the relative mRNA expression of Hes1, Nedd4 and Fbxw7 in CLL cells isolated from NSG mice fed with or without dox water for 3 weeks. (G) Western blot analysis to measure Nedd4 and Itch protein levels in NSG mice fed with or without dox. (H) Bar graph showing the relative mRNA expression of Nedd4 in four different IRF4^{-/-}Vh11 CLL samples compared to B cells isolated from wildtype B6 and IRF4^{+/+}Vh11 mice. (I) Western blot analysis to measure the levels of Nedd4 protein in IRF4^{-/-}Vh11 CLL samples compared to IRF4^{+/+}Vh11 B cells. The numbers at the bottom represents Nedd4 expression measured by densitometric analysis using ImageJ software. Actin is used as the loading control. *p value ≤0.01 **p value ≤0.05.

5.3.2 IRF4 directly binds to *nedd4* gene.

We performed Chromatin Immunoprecipitation sequencing (ChIP-seq) to identify genome wide binding sites for IRF4 in B1 cells. For this study, IRF4^{+/+}Vh11 B1 cells were used to map IRF4 binding sites and IRF4^{-/-}Vh11 B1 cells were used as control cells. The binding profile of IRF4 on the *Nedd4* locus was analyzed. Strikingly, ChIP-seq revealed a robust binding of IRF4 in the promoter region of *nedd4* gene (Figure 2A). Furthermore, the IRF4 binding peak was mapped to a region harboring a canonical Interferon-Stimulated Response Element (ISRE) represented by GAAANNGAAA DNA motif (Figure 2A). The ISRE element was present 2 kilobases (kb) upstream to the transcription start site (TSS) in the *nedd4* gene promoter (Figure 2A). IRF4 has been previously shown to bind 3' enhancer in the kappa immunoglobulin light chain locus (24). Our ChIP-seq screen showed a strong binding peak for IRF4 in the 3' kappa enhancer region, ascertaining the specificity of our assay (Figure 2B). The IRF4 binding to the ISRE motif in the *nedd4* gene locus was further confirmed by the conventional CHIP assay, which showed significant enrichment of the ISRE motif in *nedd4* gene promoter by anti-IRF4 antibody in IRF4^{+/+}Vh11 B1 cells (Figure 2C). Notably, we did not observe an enrichment of IRF4 binding at a region 4kb upstream to the TSS (Figure 2C). In summary, our results indicate that *Nedd4* is a direct target of IRF4 in B1 cells.

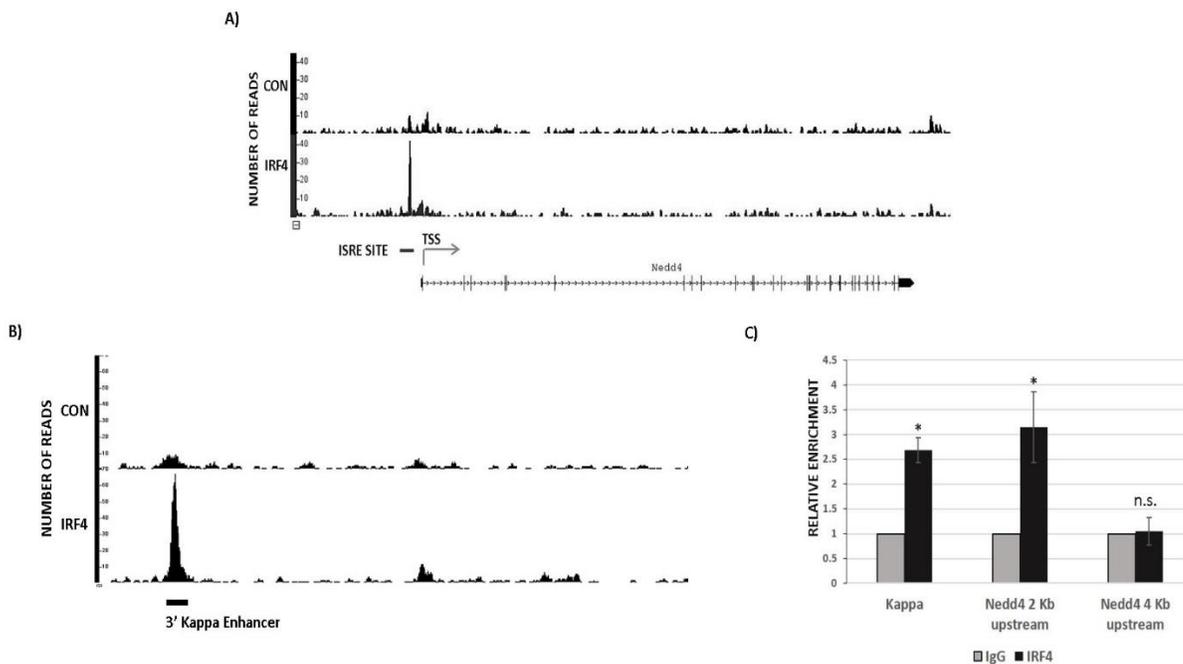


Figure 2 **IRF4 directly binds to *nedd4* gene.** (A) ChIP-seq data showing endogenous IRF4 binding at *nedd4* gene locus in B1 cells isolated from IRF4^{+/+}Vh11 mice. Immunoprecipitation of DNA fragments using anti-IRF4 antibody from IRF4^{-/-}Vh11 B1 cells is used as control. TSS represents transcription start site and ISRE represents Interferon Stimulated Response Elements located in *nedd4* gene promoter. (B) ChIP-seq data showing IRF4 binding to the 3' kappa light chain enhancer used as positive control in IRF4^{+/+}Vh11 B1 cells. (C) Bar graph representing the data from conventional ChIP assay in B1 cells using IgG and anti IRF4 antibody. Kappa represents primers spanning the 3' enhancer in the Kappa Ig light chain locus used as positive control for IRF4 binding. The data shown in (C) is representative of three independent experiments. *p value ≤0.01.

5.3.3 IRF4 regulates Nedd4 expression in B1 but not B2 cells.

A previous study has suggested that IRF4 may regulate the expression of Fbxw7 in B2 cells (11). Our results show that IRF4 regulates Nedd4 but not Fbxw7 expression in CLL cells. It appears that expression of Nedd4 and Fbxw7 may be differentially regulated by IRF4 in different B cell subsets. To clarify this issue, we decided to measure the expression of Fbxw7 and Nedd4 in B cell subsets isolated from IRF4 proficient and deficient mice. We first analyzed the levels of Nedd4 in IRF4 deficient B1 cells. Our result shows that IRF4 deficiency in B1 cells led to a significant decrease in expression of Nedd4 at the level of protein as well as mRNA (Figure 3A and 3B). The observed decrease in Nedd4 in IRF4^{-/-} B1 cells was accompanied by an increase in Notch2 expression and a corresponding increase in Notch target gene Hes1 (Figure 3A). However, compared to IRF4 proficient B1 cells we did not observe a significant change in the expression of E3 ubiquitin ligase, Fbxw7 in IRF4 deficient B1 cells (Figure 3B). We then measured the levels of Nedd4 and Fbxw7 in splenic B2 (normal B cells) cells. Surprisingly, IRF4 deficiency in splenic B2 cells was not associated with a significant change in the expression of Nedd4 (Figure 3C). Conversely, Fbxw7 levels were decreased in IRF4 deficient B2 cells (Figure 3C). These results confirm that expression of Nedd4 and Fbxw7 are differentially regulated by IRF4 in distinct B cell subsets.

IRF4 binds to DNA either as a homodimer or as a heterodimer with other transcription factors. It has been shown that DNA binding affinity of IRF4 for their target genes can be influenced by its own concentration as well as by the availability of its interacting partners (25). Pu.1 belongs to Ets family of transcription factor and is a key interaction partner for IRF4 in B cells (25). Therefore, to understand the observed discrepancy in the regulation of Nedd4 by IRF4 in B1 versus B2 cells, we assessed the levels of IRF4 and its interaction partner Pu.1. Intriguingly, the expression levels of Pu.1 were significantly higher in IRF4^{+/+} B2 cells than in IRF4^{+/+} B1 cells (Figure 3D). This finding is consistent with a previous report describing low levels of expression of Pu.1 mRNA in B1 cells (26).

The expression levels of Spi-b, which also belongs to the Ets family of transcription factors, were much lower and unaltered between B1 and B2 cells (data not shown). Interestingly, intracellular staining analysis further reveals that IRF4 was expressed at much higher levels in B1 cells than in B2 cells (Figure 3E). Collectively, these results show that IRF4 directly binds to *nedd4* gene locus to regulate its expression in B1 cells but not B2 cells and that expression levels of IRF4 and Pu.1 are distinct in B1 and B2 cells.

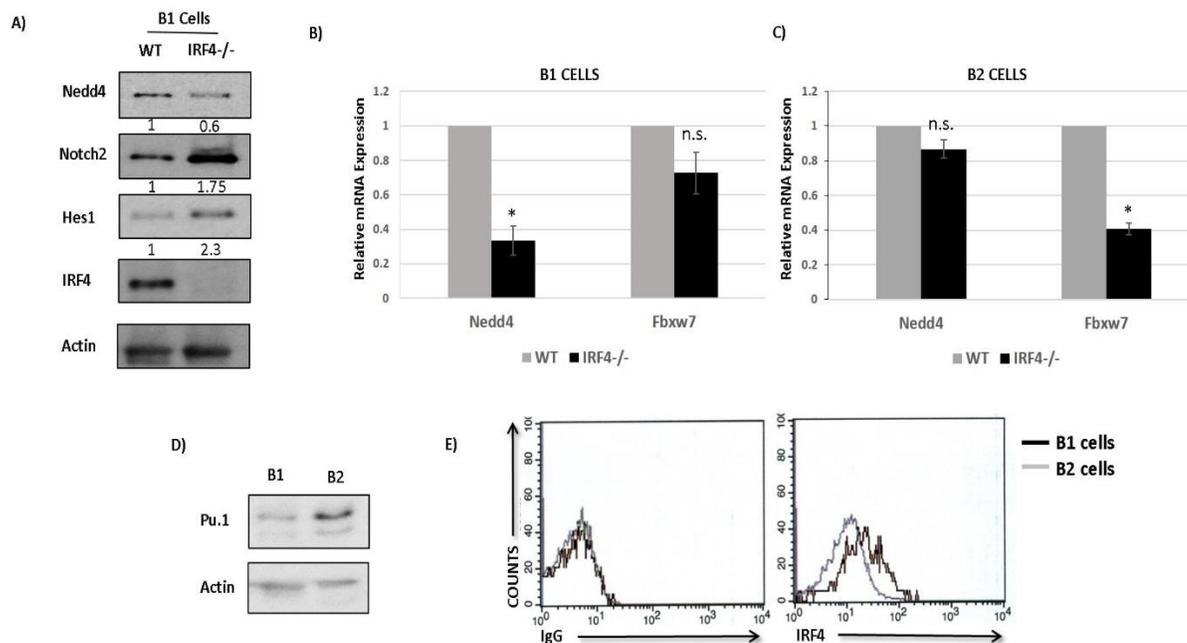


Figure 3 IRF4 regulates Nedd4 expression in B1 but not B2 cells. (A) Western blot showing the levels of Nedd4, Notch2, Hes1 and IRF4 in IRF4^{-/-} B1 cells compared to IRF4^{+/+} B1 cells. The numbers below represent normalized relative expression calculated by densitometric quantification of respective proteins. (B) Bar graph showing the relative mRNA expression of Nedd4 and Fbxw7 in IRF4^{-/-} and IRF4^{+/+} B1 cells. (C) Bar graph showing the relative mRNA expression of Nedd4 and Fbxw7 in IRF4^{-/-} and IRF4^{+/+} B2 cells. (D) Western blot analysis to detect the levels of Pu.1 in B1 cells isolated from PC and B2 cells isolated from spleen of wild type mice. (E) Flow cytometry analysis using intracellular staining to measure the levels of IRF4 in PC B1 cells and splenic B2 cells. The histograms represents intracellular staining with isotype control antibody (left panel) and with IRF4 antibody (right panel). Gray line represents B2 cells and Black line represents B1 cells. Cells were gated specifically on B1 and B2 populations based on IgM and B220 staining. *p value ≤ 0.01 .

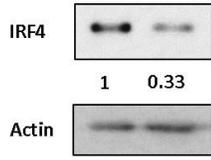
5.3.4 IRF4 regulates Nedd4 expression in human B cells and CLL cells to downregulate Notch protein.

We next wanted to determine whether IRF4 regulates Nedd4 expression in human B cells. To study this, we manipulated the levels of IRF4 using siRNA mediated knockdown in normal human B cells isolated from peripheral blood of healthy donors. Normal human B cells were nucleofected with a pool of 4 siRNAs specific to IRF4 mRNA and with a pool of 4 scrambled siRNAs as control (Figure 4A). siRNAs specific to IRF4 led to a strong decrease in the expression of IRF4 in normal human B cells (Figure 4A). Further mRNA analysis reveals that IRF4 knockdown in human B cells led to a decrease in the expression of Nedd4 and a concurrent increase in the expression of Hes1 (Figure 4B). Importantly, the levels of Fbxw7 remained unaffected by IRF4 knockdown in normal human B cells (Figure 4B).

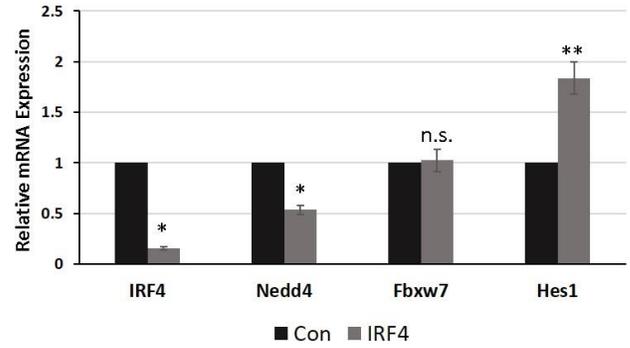
Effect of Nedd4 on Notch protein turnover has been mainly studied in drosophila. Here, we wanted to further determine whether Nedd4 can also regulate Notch protein turnover in CLL cells. We used a siRNA mediated knockdown approach to manipulate Nedd4 protein levels in human Mec-1 CLL cells. Mec-1 cells are an established human CLL cell line that expresses both Notch1 and Notch2 proteins. A pool of 4 siRNAs specific to Nedd4 mRNA were nucleofected into the Mec-1 cells, while a pool of 4 scrambled siRNAs were used as controls. Knockdown of Nedd4 for 48 hours in Mec-1 cells indeed led to an increase in the expression of both Notch1 and Notch2 proteins compared to knockdown with scrambled siRNA controls (Figure 4C). Importantly, the protein levels of Nedd4 protein family member Itch as well as IRF4 remained unaffected by Nedd4 protein knockdown (Figure 4C). Therefore, we conclude that Nedd4 can regulate Notch proteins turnover in CLL cells. In summary, our results indicate that IRF4 regulates expression of Nedd4 in human B cells to downregulate Notch receptors.

We also assessed the levels of IRF4 and Nedd4 in human CLL cells. As previously reported, the levels of IRF4 were generally lower in human CLL samples. However, some of the CLL samples predominantly belonging to patients with good prognosis based on their CD38 negativity expressed higher levels of IRF4 (Figure 4D and Table 1). Interestingly, the CLL samples expressing higher levels of IRF4, also expressed higher levels of Nedd4 (Figure 4D). The protein levels of IRF4 showed a high degree of correlation to the protein levels of Nedd4 with a regression coefficient of 0.866 (Figure 4E). Furthermore, the expression of Nedd4 in a larger cohort of CLL samples from oncomine were reduced compared to peripheral blood mononuclear cells (Figure 4F). In conclusion, these studies establish the conservation of IRF4 and Nedd4 regulatory axis in human B cells and CLL cells.

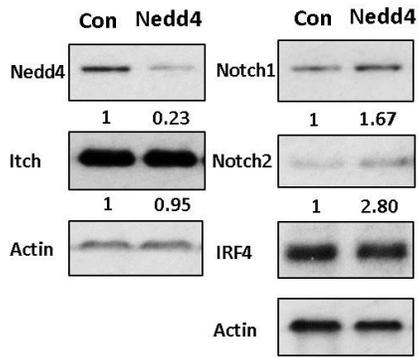
A)



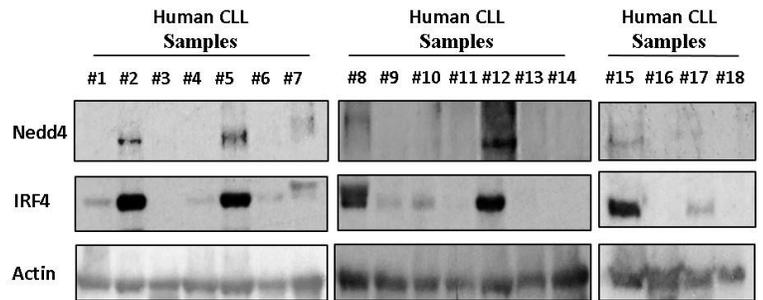
B)



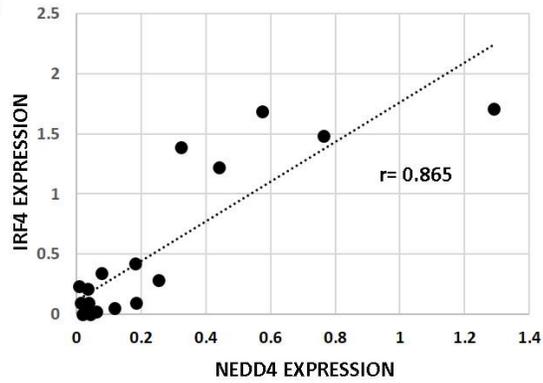
C)



D)



E)



F)

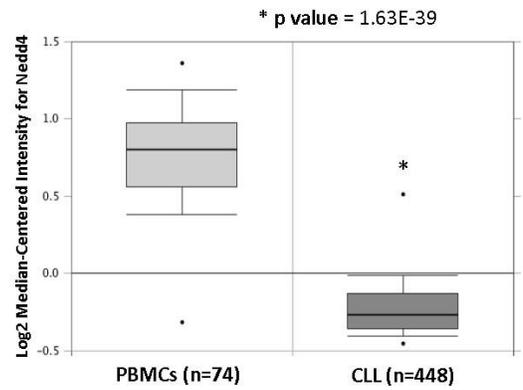


Figure 4 IRF4 regulates Nedd4 expression in human B cells and CLL cells to downregulate Notch protein. (A) Western blot showing IRF4 knockdown using IRF4 specific or scrambled control siRNA in normal human B cells isolated from healthy donors. The number below represents normalized relative expression. (B) Bar graph showing relative mRNA expression of IRF4, Nedd4, Hes1 and Fbxw7 in normal human B cells in control versus IRF4 specific siRNA. (C) Western blot analysis of Nedd4, Itch, Notch1, Notch2 and IRF4 following Nedd4 knockdown using siRNA in human Mec-1 CLL cells. Knockdown with scrambled siRNA is used as controls (con). The numbers below represent the normalized relative expression of respective genes measured by densitometric analysis. (D) Western blot analysis of Nedd4 and IRF4 expression in human CLL samples represented by each individual lane. (E) Scatter plot to show the correlation between IRF4 and Nedd4 protein expression in human CLL cells. The dotted line represents the linear trend line. Pearson coefficient (r) value is 0.866. (F) Box plot showing the Nedd4 mRNA expression in a large cohort of CLL samples compared to normal peripheral blood mononuclear cells (PBMCs). The dataset used for analysis was Haferlach leukemia dataset from oncomine. *p value ≤ 0.0001 **p value ≤ 0.01 .

5.4 DISCUSSION

Mutational and constitutive activation of Notch signaling is reported in patients without Notch mutations (8, 27). However, the molecular mechanisms leading to aberrant Notch signaling in CLL cells remain poorly defined. Our results presented here establish IRF4 as a critical regulator of Notch signaling during CLL development. Since, low levels of IRF4 is a common feature of CLL, the deregulated IRF4-Notch axis may represent a major pathway in the molecular pathogenesis of CLL. Nedd4 promotes ubiquitination and degradation of Notch receptors and has been shown to limit aberrant ligand independent activation of Notch signaling (21). Interestingly, we identify Nedd4 as a key IRF4 target gene involved in impeding the responses of CLL cells and their precursors to Notch signaling. Our studies show that expression of Nedd4 was defective in IRF4^{-/-} Vh11 CLL cells and their precursors. Additionally, we show that IRF4 directly bound to the promoter region of *nedd4* gene. Moreover, reconstitution of IRF4 induced the expression of Nedd4 in IRF4^{-/-} Vh11 CLL cells and downregulated Notch2. Notably, our data also show that Nedd4 is regulated by IRF4 in normal human B cells and can downregulate Notch proteins in human CLL cells. Intriguingly, a recent GWA study identified SNPs upstream to the *nedd4* gene locus to be strongly associated with CLL development in human patients (28). Although, the functional significance of these SNPs on Nedd4 expression remains to be determined, our *in silico* analysis using a large cohort of CLL samples showed a significant decrease in Nedd4 expression in CLL cells compared to normal peripheral blood mononuclear cells (Figure 4F). As an E3 ubiquitin ligase, Nedd4 may have many targets in CLL cells; however, our findings would indicate that Notch proteins are major targets of Nedd4 in the context of CLL development.

Our studies show that IRF4 regulates expression of Nedd4 in B1 cells but not B2 cells. This apparent paradoxical findings, we believe, can be explained by a recently proposed “kinetic control” model, which was put forth to explain the dynamic changes in the DNA binding abilities

of IRF4 at distinct B cell developmental stages (25). According to this model, the DNA binding landscape of IRF4 is influenced by the levels of IRF4 expression and the expression of its various interaction partners (25). IRF4 can hetero-dimerize with Ets family of transcription factors to bind EICE motifs (GGAANNGAAA), while upon homo-dimerization IRF4 binds to ISRE motifs (GAAANNGAAA). Notably, IRF4-Ets heterodimers binds EICE motifs with much higher affinity compared to the binding of IRF4 homodimers to ISRE sites (25). Moreover, this model may imply that binding of IRF4 homodimers to ISRE motifs may not occur efficiently in the presence of Ets transcription factors like Pu.1. Our results show that IRF4 is expressed at much higher levels in B1 than B2 cells. In contrast, Pu.1 is expressed at much higher levels in B2 cells than B1 cells. Therefore, the high levels of IRF4 and low levels of Pu.1 would lead to preferential binding of IRF4 to ISRE motifs present in *nedd4* gene promoter in B1 cells. On the other hand, higher levels of Pu.1 and lower levels of IRF4 in B2 cells may sequester IRF4 to EICE motifs and away from the low affinity ISRE motifs thereby, preventing its binding to *nedd4* gene promoter in B2 cells. Previous study has shown that *Fbxw7* mRNA is downregulated in IRF4 deficiency B2 cells (11). Our result also shows that *Fbxw7* expression was decreased in IRF4 deficient B2 cells. Surprisingly, unlike *Nedd4*, the *Fbxw7* expression was not significantly affected in IRF4^{-/-}Vh11 B1 and CLL cells. These results indicate that *Fbxw7* is not the major E3 ubiquitin ligase responsible for increased Notch receptor expression and signaling in IRF4 deficient B1 and CLL cells. It is still possible that *Fbxw7*, not *Nedd4*, is the E3 ubiquitin ligase that controls Notch activity in B2 cells. More studies are needed to determine whether *Fbxw7* is a direct target of IRF4 that regulates Notch turnover in B2 cells.

In summary, our studies presented here uncover a novel regulatory pathway that controls Notch activity and CLL development. The importance of this pathway is strongly supported by the evidence that components of this pathway IRF4, *Nedd4* and Notch are themselves frequently

targeted during CLL development and progression (2, 5, 28, 29). Therefore, deregulation of this pathway may represent a major pathogenesis step during CLL development and progression. Identification of this novel regulatory pathway not only helps us better understand the biology of CLL but could also offer new targets for diagnosis and therapeutic intervention.

5.5 REFERENCES

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CHAPTER 6

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

6.1 OVERALL CONCLUSIONS

6.1.1 Low levels of IRF4 are causally related to CLL development

The molecular pathogenesis of Chronic Lymphocytic Leukemia (CLL) is not fully elucidated. GWA study revealed high prevalence of SNPs in the Interferon Regulatory Factor 4 (IRF4) gene locus, insinuating a central role for IRF4 in the development of CLL (1, 2). In our studies here we deciphered the role of IRF4 in the development of CLL. At first, we used a naturally occurring, low penetrance model of CLL, the New Zealand Black (NZB) mouse. We bred the IRF4^{+/-} mice expressing low levels of IRF4 to the NZB mice (NZB IRF4^{+/-}), to study how low levels of IRF4 affects the pathogenesis of CLL in this model. Interestingly, our results showed that CLL development is markedly accelerated in NZB mice expressing low levels of IRF4 (3). NZB IRF4^{+/-} mice developed CLL at 4-5 months of age with 100% disease penetrance, when compared with the NZB IRF4^{+/+} mice that developed CLL at 10-12 months of age with 30% disease penetrance (3). The pathogenesis of CLL in NZB mice is linked to a SNP that interferes with the processing and functional maturation of the miRNA15a/16-1 (4). The chromosomal locus harboring miRNA15a/16-1 in humans is important for CLL development and is deleted in ~55-60% of CLL cases (5). Therefore, our studies show that low levels of IRF4 collaborate with the preexisting genetic defects in NZB mice to radically accelerate CLL development (3). However, the presence of preexisting genetic defects precluded these studies from conclusively demonstrating a role for low levels of IRF4 in CLL initiation.

To study the role of IRF4 in CLL initiation we used a different genetic approach. We used a genetically engineered Vh11 knock-in (KI) mouse that expresses a prearranged immunoglobulin heavy chain gene family, Vh11. B cells expressing Vh11 heavy chain predominantly develops into a specialized B cell subset, known as B1 cells (6). Although, the cellular origin of human CLL

remains unclear, in rodents, B1 cells are believed to be the precursors of CLL cells (7). Moreover, the molecular mechanisms regulating the homeostasis of B1 cell development are largely unknown. In adult mice, B1 cells represent only a small fraction of mature B cell population making them a difficult cellular population to study (1-5%) (8). However, the Vh11 KI mice have elevated numbers of B1 cells and therefore, we used these mice to study the role of IRF4 in the regulation of B1 cell development and how it affects the development of CLL. To this end, we bred the IRF4 deficient mice (IRF4^{-/-}) with the Vh11 KI mice to generate IRF4^{-/-}Vh11 mice. Remarkably, IRF4^{-/-}Vh11 mice developed CLL spontaneously at 100% penetrance (9). Notably, neither the IRF4 deficient mice nor the Vh11 KI mice developed CLL independently (9). Furthermore, the CLL cells arising from the IRF4^{-/-}Vh11 mice were transplantable to the immunodeficient host mice (9). These studies show that low levels of IRF4 deregulates B1 cell development and are consequently involved in the initiation of CLL. Our studies describe NZB IRF4^{+/-} and IRF4^{-/-}Vh11 mice as novel mouse models that mimics a major genetic predisposition for the development of human CLL (3, 9). Together, these studies here provide unequivocal evidence for a causal role of low levels of IRF4 in the initiation and progression of CLL.

6.1.2 Notch signaling is critical for CLL development in IRF4^{-/-}Vh11 mice

To elucidate the molecular mechanism through which IRF4 suppresses CLL development, we performed Gene Expression profiling of CLL cells derived from IRF4^{-/-}Vh11 mice. Our molecular analysis revealed the activation of Notch signaling pathway as a characteristic feature of IRF4^{-/-}Vh11 CLL cells. Moreover, our functional studies showed an important role of Notch signaling in promoting the proliferation and survival of B1 and CLL cells. These results were particularly intriguing because mutational activation of Notch signaling pathway has been identified as one of the most recurrent molecular events in CLL and is associated with poor disease outcomes (10-12).

The mutations identified in the Notch signaling pathway primarily leads to increased stability of Notch1 protein in CLL cells (10-12). Other than the mutational activation, studies have reported constitutive activation of Notch signaling pathway in human CLL cells (13). Additionally, *In vitro* studies relying on co-culture of CLL cells with stromal cells have also provided evidence for a role of Notch signaling in promoting the survival and chemo-resistance in human CLL cells (14, 15). Altogether, these studies have linked aberrant Notch signaling to the pathogenesis of CLL *in vitro*, however, whether Notch signaling is critical for CLL development *in vivo* remains unknown. Furthermore, the molecular mechanism leading to the deregulation of Notch signaling in CLL is still ambiguous.

In our studies here, we first used the IRF4^{-/-}Vh11 mice to study the role of Notch signaling in the development of CLL *in vivo*. Notch family of proteins in mammalian system consists of four Notch paralogues from Notch1 through Notch4. Our studies revealed that Notch signaling is hyperactivated as a result of upregulation of Notch2 protein expression in IRF4^{-/-}Vh11 CLL cells. Interestingly, a recent report described expansion of a specialized B cell subset, known as Marginal zone B cells in IRF4 deficient mice that was also attributed to increased expression of Notch2 protein (16). We next used genetic approaches to delete *Notch2* gene in IRF4^{-/-}Vh11 mice, to examine the contribution of Notch signaling in CLL development *in vivo*. We genetically deleted the *Notch2* gene specifically in B cells of IRF4^{-/-}Vh11 mice (CD19creNotch2^{fl/fl}IRF4^{-/-}Vh11). Notably, we observed a significant delay in the onset of CLL in CD19creNotch2^{fl/fl}IRF4^{-/-}Vh11 mice compared with CD19creNotch2^{+/+}IRF4^{-/-}Vh11 mice. Moreover, our studies further showed that *Notch2* gene is absolutely required for the generation of CLL cells in IRF4^{-/-}Vh11 mice. The CLL cells which emerged from CD19creNotch2^{fl/fl}IRF4^{-/-}Vh11 mice, invariably expressed high levels of Notch2 on their surface and were apparently derived from B1 cells that escaped cre-mediated deletion. We also generated a Tamoxifen inducible Notch2 deletion model to study the role of Notch Signaling

in maintenance of CLL cells in IRF4^{-/-}Vh11 mice (ERTcreNotch2^{fl/fl}IRF4^{-/-}Vh11). In line with the findings above, Notch2 deletion had a detrimental effect on the survival and proliferation of IRF4^{-/-}Vh11 CLL cells. Taken together, our results show that Notch signaling is important for both the initiation and maintenance of CLL in IRF4^{-/-}Vh11 mice. In summary, we provide the first *in vivo* genetic evidence for a critical role of Notch signaling in the development of CLL.

Multiple studies have linked Notch signaling to the pathogenesis of CLL, yet, till now the role of Notch signaling as well as its regulation during CLL development remained poorly understood. Notch signaling is activated upon engagement of Notch receptor with its ligand on the cell surface that ultimately leads to the translocation of Intracellular domain of Notch (ICN) to the nucleus, where it transcriptionally activates its target genes. The genetic models developed and used thus far to investigate the role of hyperactive Notch signaling in B cells *in vivo*, have overexpressed the ICN in a transgenic system (17). These models trigger supra-physiological, ligand-independent activation of Notch signaling that disrupts normal B cell development and have therefore hampered studies from drawing meaningful conclusions. On the other hand, hyperactivation of Notch signaling displayed by CLL cells derived from IRF4^{-/-}Vh11 mice represents a state that is more physiological. Hence, the IRF4^{-/-}Vh11 mice by themselves represent a unique and novel model system to study the role of deregulated Notch signaling in the pathogenesis of CLL. We used this novel strategy in our studies here to show for the first time that Notch signaling is critical for CLL development *in vivo*.

6.1.3 IRF4 regulates Nedd4 to attenuate Notch signaling for suppression of CLL development.

We next used Next Generation Sequencing approaches to decipher the mechanism by which IRF4 attenuates Notch signaling in CLL. To accomplish this, we used a doxycycline inducible model to reconstitute the expression of IRF4 in IRF4^{-/-}Vh11 CLL cells *in vivo*. We observed a decrease in

Notch2 protein expression and a concomitant attenuation of Notch signaling upon IRF4 reconstitution in IRF4^{-/-}Vh11 CLL cells. We performed RNA sequencing of IRF4^{-/-}Vh11 CLL cells reconstituted with IRF4 expression to delineate the molecular mechanism by which IRF4 controls the stability of Notch proteins. Interestingly, our studies identify an E3 ubiquitin ligase, Nedd4 that promotes the degradation of Notch proteins, as an IRF4 target gene in CLL cells and their precursors (B1 cells). Furthermore, using Chromatin Immunoprecipitation sequencing (ChIP-seq) our studies revealed direct transcriptional regulation of Nedd4 expression by IRF4 in CLL precursors. Moreover, we also observed a strong correlation between the expression levels of IRF4 and Nedd4 in human CLL cells. Interestingly, a recent GWA study has linked SNPs in the Nedd4 gene locus to the development of CLL (18). Although, the functional significance of these SNPs on Nedd4 expression remains to be determined, our *in silico* analysis using a large cohort of CLL samples showed a significant decrease in Nedd4 expression in human CLL cells. Together, these studies show that IRF4 attenuates Notch signaling in CLL cells and their precursors by directly regulating the expression of Nedd4.

Our studies here uncover a novel regulatory pathway that controls Notch activity and CLL development. The importance of this pathway is strongly supported by the evidence that components of this pathway; IRF4, Nedd4 and Notch are themselves frequently targeted during CLL development and progression (2, 11, 12, 18). Therefore, deregulation of this pathway may represent a major pathogenesis step during CLL development and progression. Identification of this novel regulatory pathway not only helps us better understand the biology of CLL but could also offer new targets for diagnosis and therapeutic intervention.

In summary, our studies here progressed our current understanding of the biology of CLL and provided strong evidence for a causal role of IRF4 in the development of CLL. In the process we developed a novel mouse model of CLL (IRF4^{-/-}Vh11) that mimics a major genetic predisposition

for human CLL. Also, the IRF4^{-/-}Vh11 mice presents the full spectrum of clinico-pathological features associated with human CLL. We use this mouse model to further provide the first *in vivo* genetic evidence for a critical role of Notch signaling in the development of CLL. We delineate the molecular mechanism that causes deregulation of Notch signaling in CLL. We establish IRF4 as an attenuator of Notch signaling that is critical to suppress CLL development. We further identify Nedd4 as an important IRF4 target gene that suppresses Notch signaling in CLL cells and their precursors.

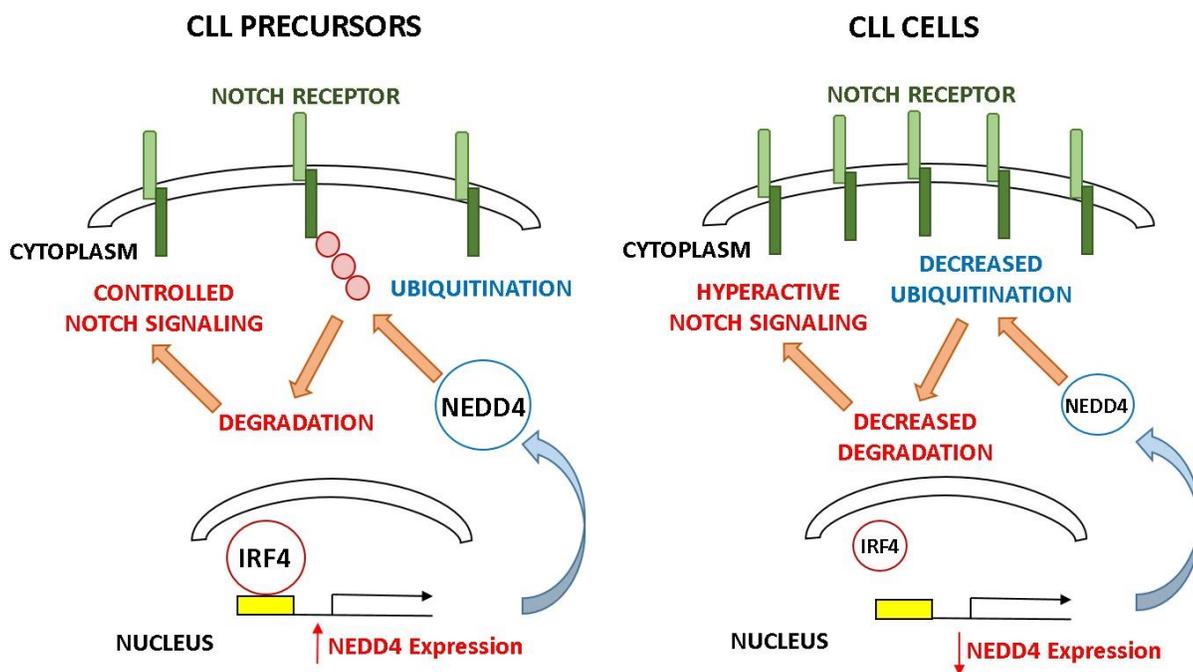


Figure 1 A diagrammatic depiction of our findings. Our studies show that IRF4 directly regulates the expression of Nedd4 in CLL precursors. Nedd4 in CLL precursors promote ubiquitination mediated degradation of Notch receptors which in turn leads to limited and controlled activation of Notch signaling. In CLL cells, SNP mediated IRF4 downregulation leads to lower levels of Nedd4. Low levels of Nedd4 leads to decreased degradation of Notch receptors and hyperactivation of Notch signaling in CLL cells.

6.2 FUTURE DIRECTIONS

6.2.1 Canonical and Non-Canonical Notch signaling in the pathogenesis of CLL

Recent studies have identified novel molecular aspects of Notch signaling that were previously unrecognized. These include, Non-Canonical features of Notch signaling that involves transcription independent roles of Notch protein that leads to activation of downstream signaling pathways in a ligand dependent or independent manner (19, 20). Although, the precise molecular mechanisms through which Non-Canonical Notch signaling operates is still not fully understood, this mode of signal transduction differs significantly from the Canonical Notch signaling (19). Canonical Notch signaling is activated upon ligand mediated proteolytic cleavage and release of ICN to the nucleus for transcriptional activation (21). Our studies here demonstrate a critical role for Notch signaling in the development of CLL *in vivo*. We genetically deleted Notch2 in mature B cells that leads to suppression of both the Canonical and Non-Canonical Notch signaling. This approach thereby prevents our studies to discriminate between the distinct roles Canonical and Non-Canonical Notch signaling may have in the pathogenesis of CLL. The distinction between the roles of these alternative aspects of Notch signaling pathway is quintessential for designing specific and effective therapies to target aberrant Notch signaling in CLL.

To this end, we are using an alternate genetic approach that involves deletion of the transcriptional regulator, Rbp-JK in mature B cells of IRF4^{-/-}Vh11 mice. Rbp-JK is the critical DNA binding component of the transcriptional activation complex assembled by ICN in the nucleus (21). Therefore, deletion of Rbp-JK would disrupts Canonical Notch signaling without affecting the Non-Canonical Notch signaling. Monitoring the pathogenesis of CLL in CD19creRbpJK^{fl/fl}IRF4^{-/-}Vh11 mice will provide valuable insights into the role of Canonical Notch signaling in CLL development. In case the pathogenesis of CLL remains unaffected in CD19creRbpJK^{fl/fl}IRF4^{-/-}Vh11

mice, it would mean that the Canonical Notch signaling is dispensable for CLL development. These results would also help in understanding the role of Non-Canonical Notch signaling in the pathogenesis of CLL.

6.2.3 The role of *Nedd4* in the pathogenesis of CLL

The role of *Nedd4* in the pathogenesis of human CLL is unexplored. Recently, a GWA study identified strong associations of SNPs upstream in the *nedd4* gene locus to the development of CLL in human patients (18). However, the functional significance of these SNPs remain ambiguous. Our *in silico* analysis using a large cohort of CLL samples from oncomine dataset revealed downregulation of *Nedd4* expression in CLL cells compared to normal peripheral blood mononuclear cells. Moreover, we identify an important role of *Nedd4* in the degradation of Notch receptor and limiting Notch signaling in B1 cells and CLL cells. As an E3 ubiquitin ligase, *Nedd4* may have several other targets as well that may affect the pathogenesis of CLL. These findings together warrant further investigation to study the role of *Nedd4* in CLL development. It will be of considerable interest to study the impact of manipulating the levels of *Nedd4* expression on CLL development and progression in *IRF4*^{-/-}Vh11 mice.

6.2.2 *IRF4* as an attenuator of Notch signaling in other B cell malignancies

Mutational and constitutive activation of Notch signaling occurs frequently in several other B cell malignancies including Mantle Cell Lymphoma, Splenic Marginal Zone Lymphoma and Diffused Large B Cell Lymphoma (10, 22-24). Therefore, it is paramount to understand the molecular mechanism that leads to aberrant Notch signaling in normal and malignant B cells. Our studies here describe an important role of *IRF4* in attenuation of Notch signaling. It is reasonable to speculate that the *IRF4* mediated attenuation of Notch signaling may play an important role in the molecular pathogenesis of other B cell malignancies as well. Together, our studies here

provide valuable insights into the molecular pathogenesis of CLL and warrant further studies to test and validate this molecular network in other B cell malignancies with deregulated Notch signaling and aberrant IRF4 expression.

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APPENDIX I

IRF4 and IRF8: governing the virtues of B lymphocytes

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Abstract Interferon regulatory factor 4 (IRF4) and IRF8 are critical regulators of immune system development and function. In B lymphocytes, IRF4 and IRF8 have been shown to control important events during their development and maturation including pre-B cell differentiation, induction of B cell tolerance pathways, marginal zone B cell development, germinal center reaction and plasma cell differentiation. Mechanistically, IRF4 and IRF8 are found to function redundantly to control certain stages of B cell development, but in other stages, they function nonredundantly to play distinct roles in B cell biology. In line with their essential roles in B cell development, deregulated expressions of IRF4 and IRF8 have been associated to the pathogenesis of several B cell malignancies and diseases. Recent studies have elucidated diverse transcriptional networks regulated by IRF4 and IRF8 at distinct B cell developmental stages and related malignancies. In this review we will discuss the recent advances for the roles of IRF4 and IRF8 during B cell development and associated diseases.

Keywords IRF4, IRF8, B lymphocytes, transcriptional regulation, leukemia and lymphoma, B cell development

Introduction

Interferon regulatory factor 4 (IRF4) and interferon regulatory factor 8 (IRF8) are highly homologous proteins that belong to the interferon regulatory factor (IRF) superfamily of transcription factors. Physiologically, IRFs are important mediators of anti-viral responses (Tamura et al., 2008). In addition to their role in antiviral responses, IRF4 and IRF8 also act as critical regulators of immune system development and function. This suggests that IRF4 and IRF8 have presumably arisen as a result of divergent evolution from a common ancestor belonging to the IRF superfamily. IRF4 and IRF8 were initially thought to be exclusively expressed in cells of immune lineages. However, recent reports have also identified IRF4 and/or IRF8 expression in melanocytes, adipocytes, smooth muscles, cardiac muscles and neurons where they perform diverse functions (Eguchi et al., 2011; Jiang et al., 2013; Praetorius et al., 2013; Guo et al., 2014; Jiang et al., 2014a; Xiang et al., 2014; Yoshida et al., 2014; Zhang et al., 2014).

IRF4 is induced in response to pathways activating NF- κ B

signaling while IRF8 is induced by type II interferon (Tamura and Ozato, 2002; Saito et al., 2007). Structurally, IRF4 and IRF8 are similar to other IRFs in having a tryptophan pentad containing DNA binding domain (DBD) and an interferon association domain (IAD) through which they can homo- or hetero-dimerize with other members of the family. To perform their transcriptional regulatory functions, IRF4 and IRF8 can form homo- or hetero-dimers with each other and with other members of the family. These homo- or hetero-dimers bind DNA with low affinity at canonical interferon-stimulated response elements (ISRE) represented as GAAANNGAAA. Besides their interaction with other IRFs, IRF4 and IRF8 can also form heterodimers with members of Ets family or AP-1 family of transcription factors (Escalante et al., 2002; Glasmacher et al., 2012; Li et al., 2012; Tussiwand et al., 2012). The heterodimers formed between IRF4/8 and Ets members, PU.1 and Spi-B bind DNA at Ets interferon composite elements (EICE) represented as GGAANN(N)GAAA. The EICE motifs were initially identified in immunoglobulin (Ig) light chain 3' κ enhancer and λ enhancer regions mediating Ig light chain locus activation (Brass et al., 1996; Brass et al., 1999). The IRF4/8-Ets hetero-dimers bind to DNA at EICE motifs with much greater affinity than ISRE motifs (Ochiai et al., 2013). More recently, IRF4 and IRF8 have been identified to co-bind DNA with AP-1 family members on AP-1-IRF composite elements (AICE) represented as GAAATGAGTCA or GAAANNNTGAGTCA in

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a variety of immune cell subsets (Glasmacher et al., 2012; Li et al., 2012; Tussiwand et al., 2012). The formation of complexes between IRF4/IRF8 with either Ets or Ap-1 transcription factors depends on the cell type and cellular context. For example, the Ap-1-IRF complexes are predominantly known to regulate cellular functions in T cells and dendritic cells while Ets/IRF complexes are critical for B cell development and functions. The cooperative binding of IRF4 and IRF8 to DNA with members of IRF, Ets and AP-1 families represents evolutionary conserved mechanisms to integrate diverse signaling inputs during immune system development and function (Glasmacher et al., 2012). Moreover, IRF4 and IRF8 have been shown to interact with transcription factors NFATs and E2A to regulate transcription in different cell types (Rengarajan et al., 2002; Hodawadekar et al., 2012).

IRF4 and IRF8 are important regulators for generation, differentiation and functions of several immune cell subsets. IRF4 play key roles in generation and functions of T follicular helper cells (Tfh), Th1 cells, Th2 cells, Th9 cells, T regulatory cells, CD8⁺ T cells, Th17 cells, macrophages and dendritic cells (Mittrücker et al., 1997; Lohoff et al., 2002; Brüstle et al., 2007; Kwon et al., 2009; Zheng et al., 2009; Satoh et al., 2010; Staudt et al., 2010; Cretney et al., 2011; Bollig et al., 2012; Tussiwand et al., 2012; Gao et al., 2013; Man et al., 2013; Persson et al., 2013; Schlitzer et al., 2013; Vander Lugt et al., 2014). Similarly, IRF8 is important for Th1, Th2, Th17, macrophage and dendritic cell development and function (Giese et al., 1997; Wu et al., 1999; Ouyang et al., 2011; Becker et al., 2012; Xu et al., 2012; Schönheit et al., 2013). In B cells specifically, IRF4 and IRF8 are expressed at multiple stages to control important decisions affecting their differentiation, function and transformation (Lu, 2008) (Fig. 1). At early stages of B cell development IRF4 functions redundantly with IRF8 to coordinate pre-B cell differentiation (Lu et al., 2003) (Fig. 1). On the other hand, at later stages of B cell development, IRF4 and IRF8 have been shown to function non-redundantly to regulate follicular versus marginal zone cell fate decisions, germinal center reaction (GC), class switch recombination (CSR) and plasma cell differentiation (Klein et al., 2006; Sciammas et al., 2006; Lu, 2008; Feng et al., 2011; Ochiai et al., 2013; Simonetti et al., 2013) (Fig. 1). In this review we will focus on describing the recent advances on the roles of IRF4 and IRF8 in B cell development and associated diseases.

IRF4 and IRF8 in early B cell development

Early pro-B cells arise from multi-potent hematopoietic progenitors in bone marrow upon coordinated expression of transcription factor E2A, Ebf1 and the B cell commitment gene Pax5 (Busslinger, 2004). An early study investigating the role of IRF8 in HSCs identified defects in early

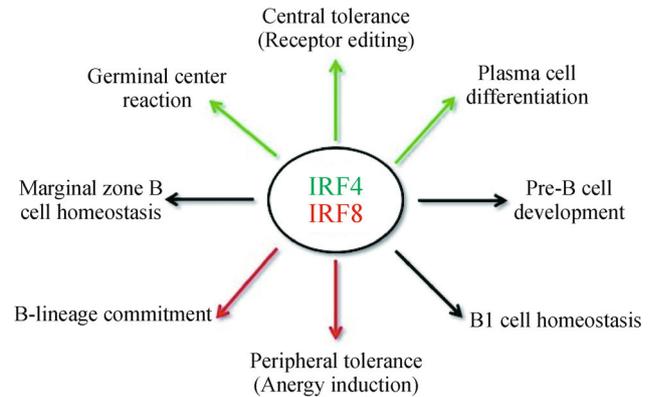


Figure 1 Regulation of B cell development by IRF4 and IRF8. The stages of B cell development regulated jointly by IRF4 and IRF8 are indicated by black arrows. Other stages of B cell development regulated exclusively by IRF4 or IRF8 are indicated by green or red arrows, respectively.

commitment to B lineage in IRF8 deficient HSCs (Wang et al., 2008). IRF8 deficiency leads to a skewed development of HSCs toward myeloid lineages at the expense of B cells (Wang et al., 2008). Furthermore, IRF8 was shown to directly bind and repress PU.1 which is known to be critical for myeloid development (Wang et al., 2008). Additionally, to reinstate B cell fate decisions IRF8 was shown to directly activate Ebf1 transcription (Wang et al., 2008). Nevertheless, HSCs can still differentiate into B lineage even in the absence of IRF8, indicating that IRF8 only modulates but is not essential for these cell fate decisions. Pro-B cells undergo immunoglobulin (Ig) heavy chain rearrangements as a first step to generate B cell receptors (BCRs). These events are mediated by several transcription factors including Pax5. IRF4 and IRF8 were initially identified as direct targets of Pax5 in early B cell development at the pro-B cell stage (Pridans et al., 2008). Intriguingly, IRF4 and IRF8 along with PU.1 have been recently identified to bind a putative enhancer region in Pax5 locus to regulate its expression (Decker et al., 2009).

At the pre-B cell stage, developing B cells undergo light chain rearrangements to generate functional B cell receptors (BCR) as they transition to the immature B cell stage. Early studies identified IRF4 and IRF8 as transcription factors that bind the 3' κ enhancer and λ enhancer to regulate the rearrangement and expression of immunoglobulin light chain at the pre-B cell stage (Brass et al., 1996; Brass et al., 1999; Ma et al., 2006). IRF4 and IRF8 are required for differentiation of pre B cells to immature B cells as B cell development is blocked at the pre-B cell stage in IRF4 and IRF8 (IRF4,8^{-/-}) double deficient mice. Furthermore, IRF4,8^{-/-} pre-B cells were shown to have a hyper-proliferative phenotype. Further analysis showed that the defects in the IRF4,8^{-/-} pre-B cells can be rescued by reconstitution of either IRF4 or IRF8 (Ma

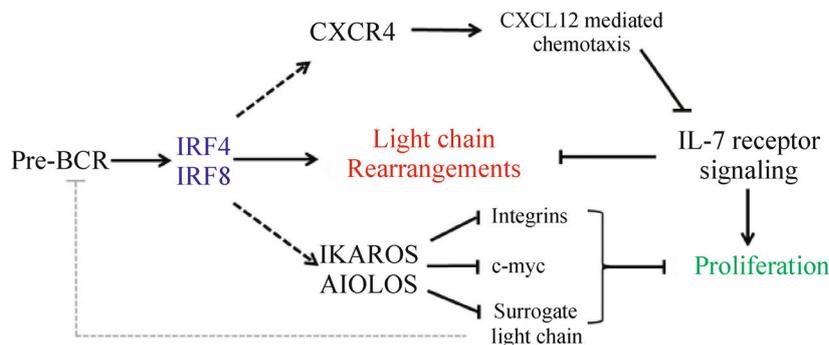


Figure 2 IRF4 and IRF8 utilize multiple mechanisms to control pre-B cell Development. Pre-BCR signaling leads to expression of IRF4 and IRF8 that directly promotes light chain rearrangement. IRF4 and IRF8 also induce Ikaros and Aiolos to inhibit pre-B cell proliferation. Ikaros directly suppresses expression of c-myc, surrogate light chain and integrins. Moreover, IRF4 regulates the expression of CXCR4, which leads to migration of pre-B cells toward CXCL12 expressing stromal cells and away from IL-7 expressing stromal cells. This leads to attenuation of IL-7 signaling.

et al., 2006). These results indicate that IRF4 and IRF8 function redundantly to orchestrate pre-B cell development.

Pre-B cell development can be further divided into two distinct stages; the cycling large pre-B cells that transition to generate resting small pre-B cells. In cycling pre-B cells a primitive BCR called the pre-B cell receptor (pre-BCR) is assembled, that functions to mediate initial expansion and eventual differentiation of large pre-B cells to small pre-B cells (Clark et al., 2014). Interestingly, IRF4 has been shown to be induced downstream to pre-BCR signaling (Thompson et al., 2007). Large pre-B cells also depend on IL-7 receptor signaling for their proliferation *in vivo*. Moreover, IL-7 has also been shown to impede pre-B cell differentiation by directly inhibiting light chain rearrangements (Mandal et al., 2011). We and others have shown that IRF4 and IRF8 limits pre-B cell expansion by negatively regulating both pre-B cell receptor and IL-7 receptor signaling (Johnson et al., 2008; Ma et al., 2008).

Expression of IRF4 and IRF8 at the pre-B cell stage occurs downstream to pre-B cell receptor signaling (Fig. 1). Upon induction, IRF4 and IRF8 further induces the expression of transcription factors Ikaros and Aiolos that functions as negative regulators of pre-B cell receptor signaling and cell cycle progression (Ma et al., 2008) (Fig. 2). Ikaros and Aiolos repress the expression of surrogate light chain (SLC), which is an essential component of the pre-B cell receptor complex (Ma et al., 2008). We further showed that Ikaros and Aiolos inhibit large pre-B cell expansion by directly binding and repressing c-myc (Fig. 2) (Ma et al., 2010). Notably, Ikaros deficient pre-B cells fail to undergo growth arrest even upon IL-7 withdrawal (Heizmann et al., 2013). These results indicate a direct role of Ikaros in inhibiting the pre-B cell expansion. Intriguingly, two recent reports have identified a novel mechanism for inhibition of cell cycle by Ikaros in pre-B cells (Joshi et al., 2014; Schwickert et al., 2014). Ikaros directly suppresses the expression of several integrins (Fig. 2) including Itga1, Itga5 and Itgb1 as well as components

mediating focal adhesions like Ptk2, Dock1 and Vcl. Therefore, increased expression of integrin and focal adhesion components in Ikaros deficient pre-B cells allow them to strongly adhere to stromal cells. The adhesion of Ikaros deficient pre-B cells to stromal cells in turn provides them with essential growth factors including IL-7 and SCF, promoting their survival and proliferation. These results suggest an indirect role for Ikaros in limiting pre-B cell expansion via inhibiting their adhesion to the stromal cells. In summary, Ikaros employs multiple mechanisms to promote pre-B cell differentiation and these mechanisms may function concurrently and may not be mutually exclusive.

IRF4 has also been shown to limit the pre-B cell expansion by attenuating IL-7 receptor signaling (Johnson et al., 2008). IRF4 was shown to regulate the expression of chemokine receptor CXCR4 in pre-B cells (Fig. 2) (Johnson et al., 2008). CXCR4 induction by IRF4 drives pre-B cells toward CXCL12 expressing stromal cells and away from the IL-7 secreting stromal cells (Tokoyoda et al., 2004). Importantly, IL-7 signaling is known to inhibit pre-B cell differentiation by directly repressing light chain rearrangements (Mandal et al., 2011). Therefore, the chemotaxis of pre-B cells to niches bearing low levels of IL-7 would be important to limit their expansion and to initiate productive light chain rearrangements (Johnson et al., 2008; Mandal et al., 2011) (Fig. 2). These results support an indirect role for IRF4 in limiting the pre-B cell expansion and promoting their differentiation.

IRF4 and IRF8 in regulation of B cell tolerance

Upon assembly of a functional B cell receptor (BCR), immature B cells exit the bone marrow and enter into the peripheral lymphoid organs. However, prior to their migration to the peripheral lymphoid organs, the BCRs of the newly generated B cells are tested for their self-reactivity. The entry of the self-reactive B cells into the periphery is abolished by

Central and Peripheral tolerance mechanisms that function at the immature and transitional B cell stages. The central tolerance mechanism in bone marrow is primarily constituted of Receptor Editing, wherein the self-reactive BCRs on newly developed B cells are edited by secondary Ig rearrangements. Self-reactive B cells that fail to surmount self-reactivity following receptor editing undergoes eventual deletion, as a default pathway. The peripheral tolerance mechanisms may render a self-reactive B cell unresponsive to antigen stimulation by a process called anergy. These tolerance mechanisms help curtail self-reactive B cells which may otherwise lead to development of systemic autoimmune diseases.

We have shown that IRF4 is involved in central tolerance mechanisms by promoting receptor editing (Pathak et al., 2008). Using a BCR transgenic mice and a membrane bound antigen we demonstrated that IRF4 is critical for secondary rearrangements at the immature B cell stage (Pathak et al., 2008). We showed that secondary rearrangement is impaired in IRF4 deficient mice. Moreover, we found IRF4 to be more critical for λ rearrangements than κ rearrangements. Consistent with our findings, a recent study also showed that elevated levels of IRF4 in pre-B cells leads to more efficient activation and rearrangements at λ locus (Bevington and Boyes, 2013). Remarkably, premature induction of IRF4 using the surrogate light chain promoter in pro-B cells was capable of triggering the entire cascade of events required for light chain rearrangements (Bevington and Boyes, 2013). Another recent report described activated NF- κ B signaling as a feature of cells engaged in receptor editing (Cadera et al., 2009). Importantly, the cells undergoing receptor editing in this study also expressed high levels of IRF4 (Cadera et al., 2009). These studies have identified a critical role for IRF4 in central tolerance mechanisms as a regulator of receptor editing.

Unlike during pre-B cell development, IRF8 cannot compensate for loss of IRF4 in receptor editing (Pathak et al., 2008). However, we have recently identified a novel role for IRF8 in regulating peripheral tolerance by maintaining the anergic state of self-reactive B cells. Using a double transgenic mouse model expressing a transgenic BCR and the secreted cognate auto-antigen, we showed that B cell anergy is breached in IRF8 deficient mice (Pathak et al., 2013). We further showed that both germline and B cell specific IRF8 deficient mice produce anti-dsDNA antibodies (Pathak et al., 2013). It is known that anergic B cells fail to undergo complete maturation and are stalled at the transitional stage. Intriguingly, we showed that IRF8 negatively regulates the survival of these anergic B cells at transitional B cell stage (Pathak et al., 2013). These studies identified IRF8 as a novel regulator of B cell anergy. However, the underlying molecular events controlled by IRF8 in maintenance of B cell anergy remain unclear. Collectively, these studies have demonstrated that IRF4 and IRF8 are critical for regulating different arms of B cell tolerance induction pathways.

IRF4 and IRF8 in Follicular, Marginal Zone and B1 B cell development

Mature B cells in mouse can be subdivided into two main subsets: the major B2 cells and the minor B1 cells. B2 cells in murine spleen can either differentiate to a predominant Follicular B cell population (FO B) or can give rise to a minor Marginal Zone B cell population (MZ B). FO B cells are primarily localized to B cell follicles situated in the splenic white pulp and are responsible for T cell dependent humoral responses. MZ B cells on the other hand, are located at the border of splenic white pulp and are required for rapid T cell independent responses against blood borne pathogens and particulate antigens. These cell fate decisions are primarily influenced by BCR specificity, BCR signaling strength and Notch signaling (Pillai and Cariappa, 2009). Both IRF4 and IRF8 have been identified as transcriptional regulators known to affect these alternative cell fate decisions in mature B cells.

In a recently published report, IRF4 was found to restrict the MZ B cell pool in a B cell intrinsic manner. IRF4 deficiency in mature B cells lead to retention of mature B cells preferentially to the splenic MZ. These defects were mechanistically attributed to the elevated levels of Notch2 protein in IRF4 deficient mature B cells (Simonetti et al., 2013). Importantly, activated notch signaling is one of the pathways obligatory required for generation and maintenance of MZ B cells (Pillai and Cariappa, 2009). Furthermore, inhibition of Notch2 by an inhibitory antibody reverses the MZ B cell defects in IRF4^{-/-} mice. Interestingly, regulation of Notch proteins by IRF4 occurs at a post-transcriptional level and is linked to the reduced levels of an E3 ubiquitin ligase Fbxw7 (Simonetti et al., 2013). Additionally, IRF4 deficiency also lead to altered expression of integrins and chemokine receptors known to mediate migration and retention of mature B cells to specific anatomical sites (Simonetti et al., 2013). It is noteworthy that the expansion of MZ B cells in IRF4^{-/-} mice can only be detected by immunohistochemistry staining but not by conventional flow cytometry analysis (Simonetti et al., 2013). This suggests that the expanded B cell population in the marginal zone of IRF4^{-/-} mice is not comprised of bona fide MZ B cells and may simply represent FO B cells that are aberrantly mislocalized.

IRF8 was also identified as a regulator of FO or MZ cell fate decisions (Tailor et al., 2008; Wang et al., 2008; Feng et al., 2011). Both germline and B cell specific deficiency of IRF8 cause an expansion of MZ B cells with a concomitant decrease in the frequency of FO B cells (Tailor et al., 2008; Feng et al., 2011). Interestingly, a BXH2 mouse harboring a point mutation in the interferon association domain (IAD) of IRF8 (R294C) phenocopies the IRF8^{-/-} mice in their MZ expansion features (Tailor et al., 2008). Although, these studies have identified a role for IRF8 in MZ B cell development, the molecular mechanism by which IRF8 restricts MZ B cell pool is still unclear.

B1 cells represent a minor B cell subset that primarily

occupies peritoneal and pleural cavities in rodents. Additionally, a small fraction of B1 cell population is also present in murine spleen. B1 cells recognize natural antigens and spontaneously differentiate to plasma cells without requiring T cell help. B1 cells are also unique in displaying properties to self-renew themselves (Hardy, 2006). On the basis of CD5 expression, B1 cells are divided into the CD5 (+) subset called B1a cells and the CD5 (-) B1b cells. IRF8 was shown to regulate the B1 cell numbers in a B cell intrinsic manner. B1b cells particularly, undergo an expansion in peritoneal cavity in the absence of IRF8 (Feng et al., 2011). Whether IRF8 regulates B1 cell numbers in peritoneal cavity by altering their self-renewal or differentiation still remains unknown. On the other hand, we have recently shown that New Zealand Black (NZB) mice expressing low levels of IRF4 (IRF4^{+/-}) exhibit defects in B1 cells. NZB IRF4^{+/-} B1 cells exhibit enhanced proliferation and survival while having decreased ability to differentiate to plasma cells (Ma et al., 2013). All of these defects cumulatively contribute to an accumulation of B1a cells in peritoneal cavities of these mice. Further studies are needed to identify the molecular events regulated by IRF4 and IRF8 in limiting the B1 cell pool.

Germinal center reaction, class switch recombination and plasma cell differentiation

Germinal centers represent specialized sites in secondary lymphoid organs that are induced during T cell dependent immune responses. Anatomically, germinal centers are constituted of a peripheral dark zone and an inner light zone. Centroblasts present in dark zone of germinal centers (GCs) represent the early arriving B cells that eventually give rise to the more mature centrocytes in the light zone. B cells in GCs undergo somatic hypermutation (SHM) to generate high affinity B cell receptors. B cells expressing high affinity BCRs bind to their cognate antigens present on antigen presenting cells (APCs) and to co-stimulatory molecules on T cells to differentiate to antibody secreting plasma cells and memory B cells. IRF4 is well defined as a transcription factor obligatory required for terminal differentiation of B cells to plasma cells (Klein et al., 2006; Sciammas et al., 2006). In GCs, IRF4 and IRF8 follow a reciprocal expression pattern (Cattoretti et al., 2006). IRF8 is shown to be highly expressed in centroblasts of GCs that are negative for IRF4 expression (Fig. 3). On the other hand, the more mature centrocytes differentiating toward plasma cells are positive for IRF4 expression (Cattoretti et al., 2006). These findings initially supported the notion that IRF4 may be dispensable for early stages of GC reaction while IRF8 may be obligatory required. In line with the notion, an early report identified severely impaired GC formation in mice globally lacking IRF8 (Lee et al., 2006). However, a recent report from the same group identified normal GC formation in mice lacking IRF8 specifically in B cells (Feng et al., 2011). These latest

findings indicate that IRF8 deficiency specifically in B cells is dispensable for GC reaction.

Contrary to IRF8, studies have shown that IRF4 is indispensable for early stages of GC reaction (Ochiai et al., 2013; Willis et al., 2014). Mice lacking IRF4 specifically in B cells fail to form GCs due to insufficient induction of Bcl6, Obf1 and AID (Fig. 3) (Ochiai et al., 2013). Bcl6 is a master regulator for GC reaction while, AID is critical for somatic hypermutation and class switch recombination (CSR) in GC B cells. Interestingly, using an inducible IRF4 transgene it was shown that a short pulse of IRF4 (for 2 days) was both required and sufficient for GC induction in IRF4^{-/-} mice (Ochiai et al., 2013). Furthermore, a recent report also identified a B cell intrinsic role for IRF4 in GC formation in response to a wide variety of antigens (Willis et al., 2014). T follicular helper (Tfh) cells represent a T cell subset that is critical for GC formation (Ramiscal and Vinuesa, 2013). Interestingly, IRF4 is recently described to be important for Tfh cell differentiation as well (Kwon et al., 2009; Bollig et al., 2012). IRF4 is shown to be critical for induction of Bcl6 that is required for generation of Tfh cells (Bollig et al., 2012). These results suggest that regulation of GC reaction may require both B cell intrinsic and extrinsic activities of IRF4.

Studies have shown that IRF4 levels undergo dynamic changes during germinal center reaction (Cattoretti et al., 2006; Ochiai et al., 2013). To rationalize these dynamic changes, Ochiai et al. have proposed a “kinetic control” model to explain how distinct stages of GC reaction are controlled by IRF4 expression (Sciammas et al., 2011; Ochiai et al., 2013). According to the “kinetic control” model, differential levels of IRF4 allow the regulation of mutually antagonistic GC and plasma cell programs in GC B cells (Ochiai et al., 2013). IRF4 at low levels co-operate with Ets and AP-1 family members to co-bind EICE and AICE motifs respectively to initiate GC program (Fig. 3). At later stages high levels of IRF4 cause a shift in binding to low affinity ISRE motifs to execute plasma cell differentiation program (Fig. 3). Several lines of evidences support the “kinetic control” model. First, in plasma cells containing low levels of Ets family members, IRF4 was shown to regulate Blimp-1 expression by binding to its locus at sites with ISRE motifs (Sciammas et al., 2006; Ochiai et al., 2013). Secondly, in plasma cells expressing high levels of IRF4, AID expression is repressed by IRF4; although IRF4 was not shown to bind AID locus (Sciammas et al., 2006). Thirdly, in GC B cells and Diffuse large B cell lymphoma cell (DLBCL) lines, IRF4 was shown to suppress Bcl6 expression by primarily binding to regions rich in ISRE motifs (Saito et al., 2007). Presumably, at lower levels in early GC B cells; IRF4 co-operate with Ets and Ap-1 family members to induce Bcl6 and AID whereas, at later stages IRF4 predominantly binds to ISRE motifs to shut down their expression. This would also explain the seemingly contradictory findings on role of IRF4 on Bcl6 expression in B cells (Saito et al., 2007; Ochiai et al., 2013).

IRF4 is induced in B cells upon BCR engagement to their

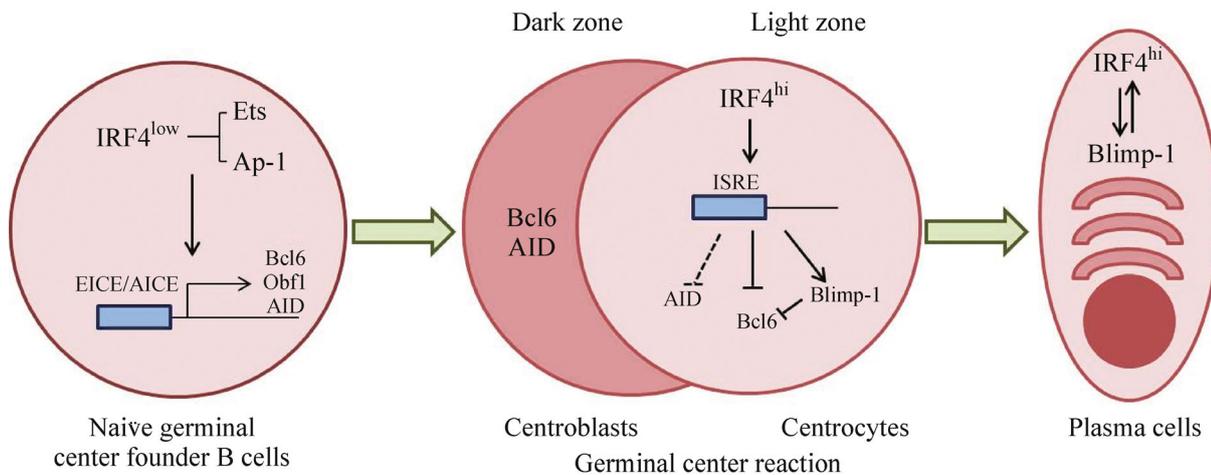


Figure 3 Role of IRF4 in germinal center (GC) reaction. At low levels, IRF4 (IRF4^{low}) binds cooperatively with Ets and AP-1 family members at EICE/AICE motifs and is critical for induction of Bcl6, Obf1 and AID to initiate GC reaction. In centrocytes located in light zone, high levels of IRF4 (IRF4^{hi}) is induced by antigen binding to high avidity BCRs. High levels of IRF4 cause a shift in binding of IRF4 from EICE/AICE motifs to low affinity ISRE motifs. IRF4 binding to ISRE induces Blimp-1 and represses Bcl6 to end GC reaction and to initiate plasma cell differentiation program.

cognate antigens and binding to co-stimulatory receptor on T cells. Presumably, the BCR signaling strength will directly determine the expression levels of IRF4. Therefore, naïve B cells expressing high avidity BCRs capable of inducing high levels of IRF4 can suppress conventional GC reaction and spontaneously differentiate to plasma cells. On the other hand, in naïve B cells expressing low avidity BCRs, a GC program is initiated due to low levels of IRF4. Low levels of IRF4 in these cells cooperate with Ets and Ap-1 family members to directly induce Bcl6 and AID expression and give rise to GC founder cells (Fig. 3). Upon ingress to the GCs, the GC founder cells in Dark Zone (centroblasts) undergo SHM events to generate high avidity BCRs. The downstream signals from the high avidity BCRs and co-stimulatory receptors on T cells can now sufficiently induce IRF4 in centrocytes to then execute a plasma cell differentiation program. To initiate a plasma cell differentiation program, high levels of IRF4 induces the expression of Blimp-1 that further suppresses Bcl6 expression to terminate GC program (Fig. 3). Thus, IRF4 levels in GC cells represent a critical checkpoint that determines the exit from a GC program and initiation of a plasma cell differentiation program. Therefore, IRF4 plays deterministic roles in initiating GC program, executing the exit from GCs and eventual plasma cell differentiation. The plausible flow of events summarized here are similar to the model proposed by Sciammas et al. in which IRF4 functions as a determinant of BCR signaling strength to direct these B cell fate decisions (Sciammas et al., 2011). It is worth noting that although initial studies identified IRF4 expression in centrocytes, subsequent studies have notably failed to detect IRF4 expression in any B cell population undergoing GC reaction (Willis et al., 2014).

Hence, it appears that IRF4 is expressed at high levels in B cells only upon exit from GCs. However, it is also possible that IRF4 is expressed only transiently or at a low level in centrocytes making it difficult to detect.

B cell malignancies and diseases

Consistent with critical involvement of IRF4 and IRF8 in B cell development, deregulated expression of IRF4 and IRF8 is associated with pathogenesis of several B cell malignancies and diseases. IRF4 is known to play distinct roles in B cell malignancies. In early B cell derived acute lymphoblastic leukemia (B-ALL) and mature B cell derived Chronic lymphocytic leukemia (CLL), IRF4 functions as a tumor suppressor (Fig. 4) (Pathak et al., 2011; Shukla et al., 2013). However, in multiple myeloma (MM) originated from plasma cells, it acts as a survival factor (Fig. 4) (Shaffer et al., 2008). The role of IRF8 in B cell related anomalies is not well studied. IRF8 is implicated as a tumor suppressor in myeloid lineage derived neoplasms while, in B cells it has been recently linked to the pathogenesis of Follicular lymphoma (FL) and CLL (Fig. 4) (Koniczna et al., 2008; Slager et al., 2011; Bouamar et al., 2013; Li et al., 2014).

B cell acute lymphoblastic leukemia (B-ALL)

B-ALL is a precursor B cell derived malignancy, predominantly affecting children and having an aggressive clinical course. Although, clinical studies have not demonstrated a clear correlation between IRF4 and B-ALL, the role of IRF4

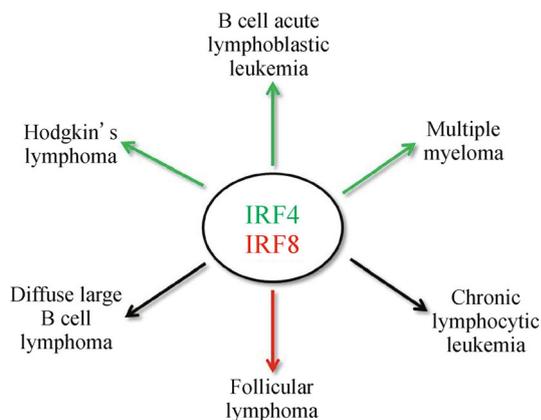


Figure 4 Role of IRF4 and IRF8 in B cell Malignancies. Green arrows indicate malignancies associated with deregulated IRF4 expression. Red arrows indicate malignancies associated with deregulated expression of IRF8. Black arrows indicate malignancies associated with deregulated expression of both IRF4 and IRF8, however; their roles in these malignancies are not essentially redundant.

has been well-studied in murine models of the disease. Consistent with its role in pre-B cell differentiation, IRF4 acts as tumor suppressor in murine models of B-ALL. Using an E μ -myc model of ALL, we have recently highlighted the importance of IRF4 in the development of ALL. IRF4^{+/-} E μ -myc mice develop ALL with an extremely short disease latency (~8 weeks) compared to the IRF4^{+/+} E μ -myc mice (~20 weeks) (Pathak et al., 2011). Similarly, IRF4 was shown to suppress the proliferation BCR-ABL1 derived mouse B-ALL clones (Acquaviva et al., 2008). Furthermore, IRF4 but not IRF8 was induced upon treatment of B-ALL cells with imatinib that inhibits the oncogenic fusion protein BCR-ABL1 (Acquaviva et al., 2008). Therefore, IRF4 functions in impeding the development and progression of B-ALL in mouse models of the disease. However, the exact molecular mechanism for tumor suppressive role of IRF4 in B-ALL needs further investigation.

Diffuse large B cell lymphoma (DLBCL)

DLBCL represents a diverse group of B cell malignancy that can be divided into several subgroups including germinal center B cell type DLBCL (GCB type) and activated B cell type DLBCL (ABC type) (Alizadeh et al., 2000). IRF4 expression is high in ABC type DLBCL, while GCB type DLBCL is negative for IRF4 expression (Cattoretti et al., 2006). However, the functional significance of IRF4 expression in ABC type DLBCL is ambiguous. Initial studies identified IRF4 as a suppressor of Bcl6 in GC B cells as well as DLBCL cell lines, where Bcl6 functions as an oncogene (Saito et al., 2007). Strikingly, DLBCL cell lines were shown to harbor mutations in the IRF4 binding sites, generating Bcl6 alleles that do not respond to IRF4 mediated suppression

(Saito et al., 2007). Consequently, these mutations allow high expression of Bcl6 in the presence of IRF4. Furthermore, the plasma cell differentiation regulator Blimp-1 is inactivated by multiple mechanisms in ABC type DLBCL (Mandelbaum et al., 2010). These findings indicate that the genetic alterations carried by ABC type DLBCL leads to an abortive plasma cell differentiation program.

IRF4 and Spi-B have been identified as survival factors for ABC type DLBCL. In these studies IRF4 functions with Ets family member Spi-B to repress type I interferon responses in DLBCL cell lines (Yang et al., 2012). IRF4 and Spi-B co-bind to EICE motifs to suppress IRF7 causing subsequent inhibition of interferon β production and interferon response mediated cell death (Yang et al., 2012). Furthermore, the drug lenalidomide was shown to inhibit the survival of ABC DLBCL cells by downregulating Spi-B and IRF4 levels. It is noteworthy that Spi-B is amplified and overexpressed in ~25% of DLBCL cases (primarily ABC type) (Lenz et al., 2007; Lenz et al., 2008). Moreover, the ABC DLBCL cell lines are shown to be sensitive to knock down of Spi-B (Lenz et al., 2008). Furthermore, IRF4 is shown to execute normal plasma cell differentiation without cooperative binding with Spi-B (Ochiai et al., 2013). Therefore, Spi-B overexpression due to genetic alterations may derail the transcriptional network critical for differentiation of activated B cells. Since IRF4 interacts with Spi-B, it is reasonable to speculate that Spi-B overexpression may alter the DNA binding landscape of IRF4 and obstruct its normal functions in plasma cell differentiation. Hence, the pro-survival effects of IRF4 observed in these studies may result from modulation of IRF4 DNA binding activity by Spi-B. IgH-IRF4 translocations are recently identified in ~4.5% of pediatric DLBCL cases further indicating an active role of IRF4 in pathogenesis of DLBCL (Salaverria et al., 2011). Surprisingly, the cases with IRF4 translocations have favorable outcomes. Nevertheless, further mechanistic insights are needed to fully delineate the role of IRF4 in the etiology of different subgroups of DLBCL.

Consistent with their GC B cell origin, IRF8 is expressed in GCB type DLBCL. A recent report identified IRF8 mutations in ~6% of follicular lymphoma cases. The mutations identified predominantly mapped to the c-terminus region of IRF8 with still unidentified functional consequences (Li et al., 2014). Similar to IRF4, IRF8 is also identified as a fusion partner with IgH locus in small subgroup of DLBCL patients (Bouamar et al., 2013). However, the precise role of IRF8 in these malignancies is still ambiguous. The identification of genetic alterations targeting IRF8 in DLBCL warrant further studies in order to elucidate its role in pathogenesis of DLBCL.

Chronic lymphocytic leukemia (CLL)

CLL is a mature B cell derived malignancy marked by

progressive accumulation of CD5⁺ CLL cells. An initial study identified higher IRF4 expression to correlate with better prognosis in CLL patients (Chang et al., 2002). More recently a genome wide association study (GWAS) identified single nucleotide polymorphisms (SNPs) in the 3' untranslated region (UTR) of IRF4 locus in sporadic and familial CLL cases (Di Bernardo et al., 2008). The SNPs in the IRF4 locus are associated with strongest susceptibility for developing CLL. The risk alleles harboring the SNPs are linked to downregulation of IRF4 and poor patient outcomes (Di Bernardo et al., 2008; Allan et al., 2010; Crowther-Swanepoel et al., 2010). Another recent study identified mutations in the DNA binding domain of IRF4 in a small subset (~1.5%) of CLL patients (Havelange et al., 2011). However, the functional consequences of these mutations in B cells remain undetermined. Recently, using two distinct genetic models we have shown that low levels of IRF4 promote CLL development. New Zealand Black (NZB) mice are natural occurring, late onset mouse model of CLL. Interestingly, low levels of IRF4 dramatically accelerated CLL development in the NZB IRF4^{+/-} mice (Ma et al., 2013). Vh11 knock-in (Vh11 KI) mice contains a pre-arranged Vh11 family Ig heavy chain inserted into the heavy chain locus and Vh11 KI mice have an expanded B1 cell population (Wen et al., 2005). Since CLL cells are derived from B1 cells in rodents; we also used Vh11 KI mice to study the effect of IRF4 on CLL development. Strikingly, IRF4 deficiency mice expressing Vh11 transgene (IRF4^{-/-} Vh11) developed spontaneous CLL with 100% penetrance (Shukla et al., 2013). Importantly, neither the IRF4 deficient nor the Vh11 KI mice develops CLL independently. These studies have established a causal relationship between low levels of IRF4 and CLL development. Further studies are needed to elucidate the molecular mechanism through which IRF4 controls CLL development.

A recent GWAS study identified SNPs in the IRF8 locus to be associated with the risk of developing CLL (Slager et al., 2011). However, unlike IRF4 the SNPs in the IRF8 locus are linked to an increased expression of IRF8 among CLL patients. Future studies are needed to fully decipher the functions of IRF8 in the etiology of CLL.

Hodgkin's lymphoma (HL)

HL is a B cell malignancy presumably derived from GC B cells. Classical HL patients accumulate Hodgkin Reed-Sternberg (HRS) tumor cells that have been shown to express IRF4, whereas the expression of IRF8 has not yet been reported (Tsuboi et al., 2000; Aldinucci et al., 2010). Contrary to the role of IRF4 in CLL, HL cells require IRF4 as a survival factor and HL cell lines are sensitive to IRF4 knockdown (Aldinucci et al., 2011). Furthermore, in HRS cells IRF4 is upregulated in response to survival signals while, IRF4 is repressed upon activation of apoptotic pathways (Aldinucci et al., 2010). A recent study categorized HL cases into two

molecular subgroups based on their strength of Myc, Notch1 and IRF4 activation (Tiacchi et al., 2012). Interestingly, the HL cases with myc activation signature were also enriched for upregulation of Notch1 and IRF4 target genes. These studies indicate a myc, Notch1 and IRF4 target genes to be active in a subgroup of HL cases potentially contributing to HL pathobiology (Tiacchi et al., 2012). Moreover, according to these studies IRF4 mediated survival of HRS cells may be important for at least a subset of HL cases. Surprisingly, a recent study identified SNPs associated with the risk for developing CLL to be also associated with HL susceptibility (Broderick et al., 2010). However, the effect of these SNPs on IRF4 expression in HL cells has not yet been evaluated. Nevertheless these studies collectively point to a pro-survival role for IRF4 in IRF4 HL however; further studies are required to elucidate the mechanistic details for the functions of IRF4 in the pathobiology of HL.

Multiple myeloma (MM)

MM is an aggressive malignancy derived from plasma cells. Consistent with their plasma cell origin, MM cells express high levels of IRF4 while their IRF8 expression is low. IRF4 was initially identified to be translocated to the IgH locus and overexpressed in MM for which it was named multiple myeloma oncogene 1 (Mum1) (Iida et al., 1997). More recently, an immunomodulatory drug lenalidomide known to target IRF4 is shown to inhibit the survival and proliferation of MM cells (Lopez-Girona et al., 2011). Given the essential role of IRF4 in plasma cell differentiation, it is rather paradoxical that IRF4 functions as a survival factor in MM. Shaffer et al. have put forward the concept of "non-oncogene addiction" to explain the role of IRF4 in MM (Shaffer et al., 2008; Shaffer et al., 2009). "Non-oncogene addiction" of IRF4 describes the unusual abilities of IRF4 to direct plasma cell differentiation on one end and to function as a survival factor for MM cells on the other end. Studies aiming to identify the molecular basis for IRF4 addiction in MM have identified myc as a direct target of IRF4 (Shaffer et al., 2008). Intriguingly, myc was also shown to positively regulate IRF4 expression in MM cells thereby establishing an autoregulatory loop between the two proteins (Shaffer et al., 2008). Other IRF4 target genes in MM cells include genes involved in glucose metabolism and ATP production that are also known to be regulated by myc (Shaffer et al., 2008). This suggests that IRF4 may also be involved in metabolic adaptations associated with myc induced transformation of MM cells. Myc was initially identified to be translocated and amplified in 16% of MM cases however, a recent report has identified ~50% of MM cases to harbor myc rearrangements (Affer et al., 2014; Shou et al., 2000). These studies indicate that myc deregulation is central to the pathogenesis of MM and IRF4 in part functions as a survival factor by directly regulating myc expression. It is possible that MM may

represent a condition in which normal plasma cell differentiation program is disrupted due to sustained myc expression. It is also worth noting that myc is overexpressed by multiple mechanisms in MM cells that do not involve IRF4 (Affer et al., 2014). This means that myc functions as an oncogene in MM cells by IRF4 independent mechanisms as well.

Besides regulating myc expression, IRF4 has been recently shown to tweak the autophagy pathways in MM cells (Lamy et al., 2013). Using a RNA interference screen, caspase-10 was found to be essential for viability of MM cells irrespective of their underlying genetic alteration landscapes. Caspase-10 along with caspase like protein (cFLIP) was demonstrated to partially cleave and inhibit the Bcl2 interacting protein (Bclaf1). Furthermore, Bclaf1 functions as an inducer of autophagy by directly binding and displacing Bcl2 from Beclin1. Interestingly, IRF4 was shown to induce the expression of caspase-10 and cFLIP in MM cells (Lamy et al., 2013). Therefore, IRF4 attenuates hyperactive autophagy induction which promotes apoptosis in MM cells. Intriguingly, the same study identified a dependence of MM cells on a basal level of autophagy for their survival. Inhibition of the basal level autophagy by targeting essential autophagic machinery in MM cells leads to spontaneous decrease in viability (Lamy et al., 2013). Therefore it appears that IRF4 only inhibits the hyperactivation of autophagy pathways in MM cells but not the basal autophagy activity.

Viral infections and associated malignancies

Kaposi Sarcoma associated Herpes Virus (KSHV) infection is associated with primary effusion lymphoma (PEL) and Epstein Barr Virus (EBV) infection is associated with several B cell malignancies including B lymphoblastoid lymphoma (LBL). Similar to the induction of IRF4 by NF- κ B signaling in normal B cells, viral proteins modulate NF- κ B signaling to regulate IRF4 expression (Forero et al., 2013). For example, the KSHV encoded viral FLICE inhibitory protein (vFLIP) induces IRF4 expression by activation of NF- κ B signaling (Forero et al., 2013). During KSHV replication, the cellular form of IRF4 (cIRF4) is inhibited by viral IRF4 (vIRF4) which subsequently suppresses c-myc expression to induce viral lytic cycle (Lee et al., 2014). Furthermore, IRF4 is expressed in PEL however; the functional significance of IRF4 expression in KSHV associated PEL is still ambiguous (Arguello et al., 2003).

A recent study has identified a role of EBV encoded EBV associated nuclear antigen 3C (EBNA3C) in counter-regulating IRF4 and IRF8 in EBV transformed cell lines. EBNA3C was found to stabilize IRF4 by direct interaction causing subsequent degradation of IRF8 in a proteasome dependent manner (Banerjee et al., 2013). Furthermore, EBV transformed lymphoblastoid cell lines showed reduced proliferation and enhanced apoptosis upon IRF4 knock-down

(Banerjee et al., 2013). IRF4 was further shown to be phosphorylated in a c-src dependent manner which interfered with the DNA binding ability of IRF4 in EBV transformed cells (Wang and Ning, 2013). Furthermore, EBNA3C was shown to bind AICE and EICE sites in p14 (ARF)/CDKN2A locus, to repress its expression (Portal et al., 2013; Jiang et al., 2014b). These studies collectively indicate that viral proteins mediated phosphorylation of IRF4 may lead to suppression of its normal cellular functions. Additionally, the EBV viral proteins may bind DNA at usual IRF4 and IRF8 binding sites to perform their oncogenic functions.

Concluding remarks

IRF4 and IRF8 act as quintessential regulators at several stages of B cell development. IRF4 and IRF8 are co-opted to execute specific transcriptional programs at certain B cell stages while, at others they independently and distinctively govern critical developmental decisions. Interactions of IRF4 and IRF8 with Ets family members, NFATs, E2A, and recently identified AP-1 family members have highlighted them as molecular rheostats integrating diverse signaling inputs (Rengarajan et al., 2002; Glasmacher et al., 2012; Hodawadekar et al., 2012; Li et al., 2012; Tussiwand et al., 2012). Moreover, the “kinetic control” model provides the molecular basis for understanding diverse effects of IRF4 on B cell development and function (Ochiai et al., 2013). Similar themes highlighting IRF4 as a determinant of T cell receptor (TCR) signaling strength involved in mediating effector T cell differentiation have also been described (Man et al., 2013; Yao et al., 2013). Recent studies have further identified genes involved in cellular metabolism and cell cycle as some of the direct targets of IRF4 in effector T cells (Shaffer et al., 2008; Man et al., 2013; Yao et al., 2013). Future studies aiming towards identifying key target genes regulated by IRF4 and IRF8 in diverse B cell malignancies will be useful to define disease pathogenesis and to design specific therapeutic interventions.

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Compliance with ethics guidelines

This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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