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Murine Models of CLL: Role of microRNA-16 in the NZB mouse model

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Summary

Mouse models are valuable tools in the study of human chronic lymphocytic leukemia (CLL). The New Zealand Black (NZB) strain is a naturally occurring model of late-onset CLL characterized by B cell hyperproliferation and autoimmunity early in life, followed by progression to CLL. Other genetically engineered models of CLL that have been developed include (NZB × NZW) F1 mice engineered to express *IL5*, mice expressing human *TCL1A*, and mice overexpressing both *BCL2* and a tumour necrosis factor receptor associated factor. The applicability to human CLL varies with each model, suggesting that CLL is a multifactorial disease. Our work with the *de novo* NZB model has revealed many similarities to the human situation, particularly familial CLL. In NZB, the malignant clones express CD5, zap-70, and have chromosomal instability and germline Ig sequence. We also identified a point mutation in the 3' flanking sequence of *Mirn16-1*, which resulted in decreased levels of the microRNA, miR-16 in lymphoid tissue. Exogenous restoration of miR-16 to an NZB malignant B-1 cell line resulted in cell cycle alterations, suggesting that the altered expression of *Mirn15a/16-1* is an important molecular lesion in CLL. Future studies utilizing the NZB mouse could ascertain the role of environmental triggers, such as low dose radiation and organic chemicals in the augmentation of a pre-existing propensity to develop CLL.

Keywords

mouse models of CLL; NZB; microRNA

Introduction

The development of chronic lymphocytic leukemia (CLL) in humans is believed to be polygenetic, exhibiting the highest incidence of familial leukemia, yet emerging from many factors (Caporaso, *et al* 2004, Ishibe, *et al* 2001, Sellick, *et al* 2006). Molecular cytogenetic abnormalities associated with CLL have been reported (Stilgenbauer and Dohner 2004), and include chromosome duplications (Dohner, *et al* 2000, Juliusson, *et al* 1990, Molica, *et al* 1995) and deletions, particularly 13q14 deletions (Dohner, *et al* 2000, Juliusson, *et al* 1990). Mouse models with known genetic backgrounds allow for the development of CLL murine

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models, which serve as valuable tools, not only in understanding disease mechanisms, but also in evaluating dietary/environmental triggers and efficacy of novel therapies.

Mouse Models of CLL

This article presents a comparison of several mouse models of CLL (Table 1). With the exception of the New Zealand Black (NZB) model, all the models are induced by the expression of exogenous genes. The NZB mouse model of CLL, in contrast, is a *de novo* model that has been studied extensively as a model to investigate both autoimmune diseases, such as systemic lupus erythematosus (SLE), as well as B cell lymphoproliferative disorders (Manohar, *et al* 1982,Raveche, *et al* 1981,Stall, *et al* 1988,Theofilopoulos 1996). Autoreactivity has also been associated with CLL (Barcellini, *et al* 2006,Lugassy, *et al* 1992), and the NZB model displays mild autoimmunity that is associated with B cell hyperactivity, resulting in autoimmune hemolytic anemia and anti-nuclear autoantibodies.

The fundamental question is whether any mouse model faithfully depicts human CLL (Dennis 2006). There are a number of differences between mice and humans that need to be taken into account when employing a mouse model of CLL. First, the basal metabolic rate of humans is 7 times lower than mice, and humans are 3000 times larger than mice and undergo more divisions. Despite this, the overall rate of cancer development in aged mice and humans is roughly the same (about 30%); however, mice develop more lymphomas/sarcomas and humans develop more carcinomas. Mice have been good models of human disease, in that candidate human oncogenes trigger CLL in transgenic mice (particularly studies in which the expression of the candidate gene was limited to B cells), deletion of tumor suppressor genes in germline cells leads to tumor susceptibility in mice, and inactivation of oncogenes in already-formed tumors causes tumor regression in mice. Although mice have been used to test the efficacy of drugs, only about 11% of potential anti-tumor drugs in mice are employed in humans (Dennis 2006). The use of a xenograft model, in which human CLL cells are grown in immunocompromised mice (including further modification in the mice so that they express human genes), might be more useful in uncovering therapies rather than in understanding genetic mechanisms responsible for disease development.

New Zealand Mouse Models

The New Zealand mouse models are often used in studying CLL. Aged New Zealand White (NZW) mice exhibit an expansion of $CD5^+$ B-1 cells (Hamano, *et al* 1998) and aged NZB mice also exhibit clonal expansion of B-1 cells that resemble human CLL (Raveche 1990). These two strains are also commonly used to produce the (NZB×NZW)F1 hybrid model that spontaneously develops an autoimmune disease similar to human SLE (Tokado, *et al* 1991) These mice develop higher titres of anti-DNA autoantibodies but have a lower incidence of B cell malignancies than the parental NZB and NZW strains. Studies have provided evidence that different MHC haplotypes in these mice may be responsible for the development of an SLE or CLL-like phenotype. H-2-congenic mice established from NZB, NZW, and (NZB×NZW)F1 mice showed that $H\text{-}2^{d/z}$ heterozygosity in all three strains predisposed mice to develop an SLE-like disease characterized by high levels of anti-DNA autoantibodies (Hamano, *et al* 1998, Hirose, *et al* 1990). Contrary to this, $H\text{-}2^{z/z}$ homozygous mice developed CLL characterized by accumulation of IgM^+ $CD2^+$ $CD5^+$ $CD19^+$ B-1 cells in peripheral blood and lymphoid organs (Hamano, *et al* 1998, Okamoto, *et al* 1993). Homozygous $H\text{-}2^{z/z}$ NZB mice also showed much higher frequencies of B-1 cells in the peripheral blood than natural $H\text{-}2^{d}$ mice (Hamano, *et al* 1998), providing strong evidence that major histocompatibility complex (MHC) haplotypes may be a critical genetic element in the development of disease manifestations in these mice.

The NZB Model of CLL

Our laboratory has an ongoing interest in the proliferative disease processes of NZB mice, particularly in the genetic basis of this process (Raveche, *et al* 1981, Raveche, *et al* 1988, Raveche, *et al* 1992, Raveche, *et al* 2007, Raveche and Tjio 1991, Raveche, *et al* 1979). The NZB strain is a naturally-occurring model of late-onset CLL (Phillips, *et al* 1992). This strain is characterized by B cell hyperproliferation and autoimmunity restricted to IgM anti-erythrocyte and anti-DNA autoantibodies early in life followed by progression to CLL. Analysis of crosses of NZB mice with control strains indicated that multiple genes controlled the development of B lymphoproliferative disease and inheritance of high levels of interleukin (IL)-10 was correlated with the development of malignant B-1 cells (Ramachandra, *et al* 1996). Thus, in NZB, the development of CLL is in an environment of chronic antigen stimulation and abnormal immune regulation, including a cytokine imbalance. The *de novo* NZB model has many similarities to the human situation, particularly familial CLL. In the NZB, the malignant clones have CD5 expression, chromosomal instability, increased MHC Class II, zap-70 expression and germline Ig sequence. The NZB mouse has been studied extensively as a model to investigate autoimmune diseases, such as SLE (Raveche and Steinberg 1979, Theofilopoulos 1996), as well as a model for the B cell lymphoproliferative disorder, CLL (Phillips, *et al* 1992). In both the NZB and human CLL, the disease is late-appearing and, similar to a subset of CLL patients, NZB mice develop autoimmune hemolytic anemia (AIHA). As NZB mice age, they develop a monoclonal lymphoproliferative expansion characterized by increased numbers of $CD5^+$ $B220^{dull}$ B cells that are hyperdiploid (Raveche, *et al* 1979) (Figure 1). In addition, the NZB malignant $CD5^+$ B clones frequently have increased IL-10 production, and development of CLL-like clones is associated with elevated IL-10 in crosses of NZB and DBA/2 mice (Ramachandra, *et al* 1996). NZB mice engineered to be devoid of IL-10 develop CLL, but the disease differs from normal NZB in that progression is retarded (Czarneski, *et al* 2004). This and other studies suggest that one of the roles of IL-10 in the NZB model of CLL is to protect the malignant B cells from undergoing apoptosis.

During the course of our studies, we isolated an immortal cell line from NZB that has the phenotype of a malignant $CD5^+$ B-1 cell (Peng, *et al* 1994). This NZB malignant B cell line, LNC, has been a valuable tool in studying CLL. LNC can be analyzed *in vitro* for molecular abnormalities that may be associated with the development of CLL. Recently, we have found that the LNC cells, in addition to having a cytokine imbalance, increased activation markers, and failure to respond to fludarabine, also have abnormal microRNA expression profiles. LNC can also be transferred to mice and induce the development of a CLL-like disease. We have used this cell line in NZB × DBA/2 F1 recipient mice and have shown that antisense IL-10 administration *in vivo*, via mini-osmotic pump delivery or in lipid chochleates, prevents the development of CLL (McCarthy, *et al* 2004, Peng, *et al* 1995).

DNA Repair Defect in NZB Mice

DNA repair defects have been associated with the development of cancer and may underlie the development of CLL (Bouley, *et al* 2006). Nearly all CLL patients have chromosomal instability (Dohner, *et al* 2000) and increased *BCL2* expression (Hanada, *et al* 1993). Bcl-2 has been found to downregulate DNA repair, both homologous and mismatch repair (Youn, *et al* 2005). Indeed, the insensitivity of some CLL patients to alkylating agents and gamma irradiation has been found to be associated with an increase in chromosomal structural aberrations when compared to CLL patients sensitive to these treatments (Youn, *et al* 2005). Early studies in our laboratory found that DNA double strand breaks resulting in sister chromatid exchange were decreased in NZB spleen cells relative to control strains of mice (Raveche, *et al* 1979). Other studies have also found that NZB fibroblasts had reduced colony forming ability, increased chromosome abnormalities after UV irradiation (Reddy

and Fialkow 1980), and decreased the capacity to perform unscheduled DNA synthesis (Paffenholz 1978). The NZB fibroblasts also have an inefficient repair phenotype in response to X-irradiation detected as an increase in chromatid breaks (Potter, *et al* 1988). Telomeres are chromosome-capping structures that protect chromosome ends against fusions and are linked to the DNA damage signaling pathways. We have found that telomere dysfunction, which has also been observed in human CLL (Trentin, *et al* 1999) (Damle, *et al* 2004), is also present in the NZB mouse model (Peng, *et al* 1998). Increased telomerase and short telomeres are associated with shorter overall survival and progressive CLL (Ishibe, *et al* 2002) and increased telomerase was observed with disease progression in NZB mice (Peng, *et al* 1998). In both human CLL and the NZB murine model, an underlying DNA repair defect and overexpression of bcl family members may play a role, not only in the development of CLL, but also in the resistance by the malignant cells to killing by DNA damaging agents due to a failure to undergo apoptosis.

IL5 transgenic mouse model

In vitro studies have shown that B-1 cells from (NZB×NZW)F1 mice are hyper-responsive to IL-5, producing large amounts of IgM in its presence (Herron, *et al* 1988, Kanno, *et al* 1993). IL-5 is a growth factor whose receptor is preferentially expressed on $CD5^+$ B-1 cells(Hitoshi, *et al* 1990). (NZB×NZW)F1 mice congenic for an *IL5* transgene were created to examine the effects of IL-5 on SLE (Wen, *et al* 2004). Contrary to expectations, (NZB×NZW)F1- *IL5* transgenic mice showed a significant reduction in both IgM and IgG anti-DNA autoantibodies at 5 and 10 months of age as well as a reduction in lupus nephritis and incidence of proteinuria. By 15 months, all of the wild-type mice died of lupus nephritis compared to only 20% of IL-5 trangenics. On the other hand, these mice exhibited a higher frequency of B-1 cells than non-transgenics beginning at 5 months of age. By 13 months, 40% of the mice exhibited a B-1 frequency over 80% and developed CLL identified by expanded IgM^+, $CD5^{dull}$ $B220^{dull}$ cells with no significant decrease in the absolute number of T cells, B-2 cells and a population of $CD5^-$, $B220^{dull}$ B cells (Wen, *et al* 2004). *In vitro* studies showed that B-1 cells from transgenic mice had lower production of IL-5 induced and spontaneous anti-DNA IgM and IgG autoantibodies, and also had higher proliferation responses to IL-2 than non-transgenics (Wen, *et al* 2004). This model shows that overexpression of *IL5* shifts the balance between autoimmune disease and B cell malignancy, creating an optimal environment for the development of CLL and suggests that abnormalities in differentiation and proliferation of B-1 cells may provide another link between autoimmune disease and CLL.

TRAF2DN/Bcl-2 double transgenic mouse model

Expression of two physically-linked microRNAs, *MIRN15a* (*mir-15a*) and *MIRN16-1 (mir-16-1)* has been shown to be dramatically reduced in the majority of CLL cases (Calin, *et al* 2002). These two microRNAs negatively regulate *BCL2* expression (Cimmino, *et al* 2005), and downregulation of these microRNAs, coupled with the associated upregulation of *BCL2*, may contribute to the pathogenesis of CLL (Pekarsky, *et al* 2007). Overexpression of *Bcl2* in mouse lymphoid cells led to polyclonal expansion of B cells and prolonged B cell survival *in vitro*, but fell short of developing any tumor phenotype (Katsumata, *et al* 1992). A second transgenic mouse model overexpressing an isoform of *TRAF2*, (tumour necrosis factor [TNF] receptor-associated factor 2) a mediator of nuclear factor (NF)-κB and c-Jun N-terminal kinase (JNK) activation, developed lymphadenopathy and splenomegaly and showed increased levels of B cells, but failed to develop a significant malignancy (Lee, *et al* 1997, Pekarsky, *et al* 2007). Taking advantage of these two previously developed models, a bcl2-driven model of CLL was created (Zapata, *et al* 2004). *TRAF2DN/Bcl-2* double transgenic mice expressing human *BCL2* and a *TRAF2* mutant in the B cell lineage were created on a Balb/c × FVB/N background. Over time, these mice developed severe

splenomegaly with spleens 3–4 times larger than wild-type mice at 10–16 months. Death occurred as early as 6 months with a survival rate of less than 20% by 14 months, whereas single transgenics lived a normal lifespan. The majority of mice that died prematurely had spleens twice as large as asymptomatic mice and 10 times larger than the wild type. These mice developed severe lymphadenopathy and about half the mice studied also developed ascites (Zapata, *et al* 2004). Premature death was also associated with an expansion of $B220^{dull}$, IgM^{bright}, IgD^{dull}, $CD21^{dull}$, $CD23^{-}$, $CD11b^{dull}$ in the spleen. CD5 was also expressed in 75% of the mice studied, indicating a B-1 phenotype, with high accumulation of B-1 cells also seen the in blood. Accumulating B cells showed a single J_H rearrangement, revealing a single B cell clone in the spleens of mice that had developed disease, which was not observed in asymptomatic mice or single transgenics. In addition, expanded B cells from these mice demonstrated a reduced rate of spontaneous apoptosis and resistance to apoptosis induced by chemotherapeutic drugs, implying that resistance to apoptosis rather than deregulation of proliferation is responsible for the B cell accumulation in these mice. The elevated disease manifestations seen in the *TRAF2DN/Bcl-2* double transgenic mice as compared to *BCL2* or *TRAF2DN* single transgenics suggest a cooperation between bcl2- and traf-related pathways in the development of CLL.

TCL1A transgenic mouse model

Several studies have shown that expression of the T-cell leukemia/lymphoma gene (*TCL1A*) is critical in the pathogenesis of CLL (Pekarsky, *et al* 2007) and high expression of *TCL1A* correlates with the aggressive phenotype in human CLL (Herling, *et al* 2006). *TCL1A* is primarily expressed in B cells in all stages of B cell development except mature B cells. Transgenic mice that overexpress *TCL1A* in both B and T cells develop a leukemia similar to human CLL with an accumulation of $B220^{+}$ $CD5^{+}$ malignant B cells visible in year-old mice (Hoyer, *et al* 2002). The Eμ-*TCL1A* transgenic mouse was generated on the B6C3 background with the expression of *TCL1A* under the control of a V_H promoter-Ig heavy chain Eμ enhancer that becomes active at the late pro-B cell stage of the B cell development and targets expression to immature and mature B cells (Bichi, *et al* 2002, Pekarsky, *et al* 2007). In this model, *TCL1A* expression was limited to the spleen and bone marrow with high levels of expression in resting B cells that was 2.5-fold higher in $CD5^{+}$ cells. Flow cytometry revealed the presence of a IgM^{+} $B220^{dull}$ population that coexpressed CD5 and CD11b by 6 months of age in 100% of the transgenic mice despite the absence of any sign of disease (Bichi, *et al* 2002). As early as 2 months of age, a population of IgM^{+} $CD5^{+}$ B cells could be identified in the peritoneal cavity, which could be seen in the spleen by 4 months and identified in the bone marrow by 8 months of age. By 8 months, transgenic mice also displayed a 1.5 fold larger spleen, increased marginal zone, and 50–100 fold increase in cellularity in the peritoneal cavity compared to non-transgenic mice. Analysis of sorted IgM^{+} $CD5^{+}$ B cells from the spleens and peritoneal cavities of 7 month-old mice showed low proliferative activity in transgenic mice and accumulation of clonal B lymphocytes blocked in the early phases (G0/G1) of the cell cycle (Bichi, *et al* 2002). By 13–18 months, all of the mice became visibly ill with severe splenomegaly and enlarged liver associated with high WBC counts and advanced lymphadenopathy (Bichi, *et al* 2002). The tcl1 protein was observed in lymphocytes from the spleen, liver, and lymph node with clonal IgM^{+} $CD5^{+}$ B cells identified in these tissues; however, these tissues were not affected the same way in all of the mice studied. In addition, other tissues that were affected, including salivary glands, kidneys, heart, esophagus, thymus, trachea, and thoracic cavity differed between mice. Interestingly, 30% of the mice studied developed secondary malignancies in the form of solid tumors at 11–12 months of age that were not seen until at least 20 months in control mice. (Zanesi, *et al* 2006) Secondary malignancies are also common in occurrence in human CLL (Robak 2004) where CLL patients demonstrate a 20% increase in the risk of developing secondary cancers (Hisada, *et al* 2001). This similarity between human CLL and

the disease presented in the mouse model further supports the value of the Eμ-*TCL1* transgenic mouse in studying CLL.

TNFSF13 (APRIL) transgenic mouse model

TNFSF13, a proliferation-inducing ligand, is a TNF-like ligand with the ability to stimulate tumor cell proliferation *in vitro* (Medema, *et al* 2003). A large fraction of CLL tumors overexpress *TNFSF13*, and a significant increase in the levels of the gene product, april, has been observed in the serum of human B CLL patients, indicating its possible role in the development of B cell malignancies (Planelles, *et al* 2004). The *TNFSF13* transgenic mouse, backcrossed on a C57Bl/6 background, expresses *TNFSF13* under control of a T cell specific *lCK* promoter (Stein, *et al* 2002). Overexpression of *TNFSF13* in T cells leads to elevated levels of april in the serum of transgenic mice. T cells derived from these mice show significantly increased proliferation and survival upon growth factor deprivation *in vitro* along with a 2-fold increase in bcl2. Increased percentages of mature B cells can be detected in the peripheral lymph nodes but are not reflected in absolute number of cells. It is likely that this increase is observed as a product of a reduced number of T cells in peripheral lymph nodes, resulting from decreased CD62L expression and diminished T cell homing capacity(Stein, *et al* 2002). B-1 cells from *TNFSF13* transgenic mice also showed no increase in proliferation but demonstrated an increased survival ability *in vitro*, as well as in response to treatment with death agonists as compared to B-1 cells in non-transgenic mice (Planelles, *et al* 2004). The *TNFSF13* transgenic mouse develops a much milder disease compared to other models discussed with no increase in size or weight of secondary lymphoid organs or effect on life span. Disease characterized by the expansion of IgM^+ $CD5^+$ $B220^+$ cells was observed in only 40% of *TNFSF13* transgenic mice compared to 100% of mice in the *TCL1A* or *TRAF2DN/Bcl-2* models (Pekarsky, *et al* 2007). Spleen hyperplasia was detected in diseased mice with spleen architecture highly disorganized to the point that the red and white pulp areas were indistinguishable. These diseased mice also developed tumors originating from expansion of the peritoneal B-1 cell population at 9–12 months that metastasized and infiltrated non-lymphoid tissues such as mucosa, kidney, and liver. In summary, *TNFSF13* transgenic mice develop a mild expansion of splenic B-1 cells and do not die prematurely (Planelles, *et al* 2004). This model shows a role for *TNFSF13* acting systemically in these mice, affecting B-1 cell proliferation and survival and promoting B cell malignancy, but suggests overexpression of *TNFSF13* alone is insufficient for the onset of a full malignancy.

MicroRNAs in Cancer

MicroRNAs (miRNAs), representing between 1–3% of all eukaryotic genes (Bartel 2004), are small, endogenous non-coding RNAs that negatively regulate the expression of many genes (reviewed in (He and Hannon 2004)) usually by binding to the 3'UTR of the target genes via regions of sequence complementarity (Huang, *et al* 2006, Kruger and Rehmsmeier 2006, Liu, *et al* 2006). The miRNAs are first transcribed as a large primary transcript (pri-miRNA) that is then cleaved into a stem-loop precursor (pre-miRNA) and processed into the mature short miRNA form, mediated by the flanking sequences (Parizotto, *et al* 2004). In many cancers, the sequence of miRNAs, copy number, or alterations in miRNA-associated genes (*DICER and argonaute genes)*, have been found (Zhang, *et al* 2006), and the level of miRNA production is under tight control by upstream regulatory sequences (Ohler, *et al* 2004), including TATA-box sequences (Xie, *et al* 2005) and other transcription binding motifs (Megraw, *et al* 2006, Taganov, *et al* 2006).

Eμ-mmu-miR-155 transgenic mouse model

Several microRNAs are known to be involved in cancer by acting as either oncogenes or tumor suppressors (Fulci, *et al* 2007, He, *et al* 2005, Johnson, *et al* 2005). One such

microRNA, miR-155 has been shown to be upregulated in B cell lymphomas and mature B-cells (Eis, *et al* 2005, Fulci, *et al* 2007, Metzler, *et al* 2004). A microRNA profile of 56 CLL patients showed *MIRN155* to be overexpressed in almost every patient analyzed, suggesting that overexpression of *MIRN155* is a general characteristic of CLL and not a phenomenon observed in a subclass of patients (Fulci, *et al* 2007). Not surprisingly, vast overexpression of *MIRN155* under the Eμ-*myc* promoter resulted in the development of B cell malignancy in mice (Costinean, *et al* 2006, Lawrie, *et al* 2007, Rodriguez, *et al* 2007, Thai, *et al* 2007). Transgenic mice carrying a *MIRN155* transgene were created as a model for CLL. In the Eμ-mmu-miR-155 transgenic mouse model, generated on C57Bl/6 and FVB/N backgrounds, expression of *MIRN155* is under the control of a V_H promoter-Ig heavy chain Eμ enhancer that becomes active at the late pro-B cell stage of the B cell development. These mice develop splenomegaly that can be detected as early as 3 weeks of age, identified by a spleen/body weight ratio 3–4 times greater than that of wild-type mice, which did not change with age. Additionally, histological analysis revealed a consistent atypical lymphoid population invading and expanding the red pulp (Costinean, *et al* 2006). At 3 months of age, Eμ-mmu-miR-155 transgenic mice are leucopenic, having WBC counts of only 25% of wild-type controls and that decrease to around 15% by 6 months. Flow cytometry identified an expansion of lymphoid cells in transgenic mice with a phenotype ($B220^{dull}$,$CD10^{-}$, IgM^{-}, $CD5^{-}$, TCR^{-},$CD43^{-}$) that resembled human acute lymphoblastic leukemia or lymphoblastic lymphoma. These expanded cells were mainly diploid, polyclonal and did not have any consistent chromosomal abnormalities, which is uncommon in malignancies (Costinean, *et al* 2006). Nevertheless, this model demonstrates a role for miR-155 in the development of B cell malignancies and suggests that miR-155 is directly involved in the induction of polyclonal B cell expansion.

Linkage of CLL to a mutation in microRNA *Mirn16-1* loci in NZB mice

In order to determine loci linked to the development of CLL in NZB, a genome-wide linkage scan of the NZB loci associated with lymphoma/leukemia was conducted in F1 backcrosses of NZB and the DBA/2 control strain. Of 202 mice phenotyped for the presence or absence of B cell lymphoproliferative disease (LPD), surface maker expression, DNA content and microsatellite polymorphisms, 74 had disease. The $CD5^{+}$, IgM^{+}, $B220^{dull}$, hyperdiploid LPD was linked to three loci on chromosomes 14, 18 and 19, which are distinct from previously identified autoimmunity-associated loci. The loci linked to lymphoproliferative disease on the NZB chromosome 14 has synteny with human 13q14, which is not unexpected as up to 50% of cases of CLL have a loss of human 13q14.3 (Bullrich, *et al* 2001, Corcoran, *et al* 1998, Liu, *et al* 1997). In the mouse chromosome 14 region and the human 13q14 region of synteny, there are two microRNAs (miRNAs) found in the intronic region of *Dleu2, Mirn15a* and *Mirn16-1*.

The region of synteny with mouse *D14mit160* is the human 13q14 region associated with CLL, containing *MIRN15a/16-1*, and so DNA sequencing of this region in NZB was performed. The sequence of DNA from multiple NZB tissues identified a point mutation in the 3' flanking sequence of the precursor microRNA, *Mirn16-1* (Figure 2), similar to the C->T point mutation found in the *MIRN16-1* 3' flanking region in some CLL patients (Calin, *et al* 2005). This mutation was not present in other strains, including the nearest neighbor, NZW (Raveche, *et al* 2007). Mutations and alterations in miRNAs have been linked to many human cancers (Sevignani, *et al* 2006). In leukemias, in particular, alterations in miRNAs have been found, and in human B-CLL, the most frequently deleted genomic region contains *MIRN15a* and *MIRN16-1* and mutations in this region have been reported (reviewed in (Calin, *et al* 2006)). Recent studies have also shown a significant association between the chromosomal location of miRNAs and those of mouse cancer susceptibility loci that influence the development of solid tumors (Sevignani, *et al* 2007).

Analysis of the levels of mature miR-16 in tissues isolated from NZB mice revealed decreased levels of the microRNA in NZB lymphoid tissue (Figure 2). The delivery of exogenous miR-16 to our NZB malignant B-1 cell line resulted in cell cycle alterations, including a decrease in cells in S phase and a G1 arrest (Figure 3). Linkage of the *Mirn15a/16-1* complex and the development of B-LPD in this spontaneous mouse model suggest that the altered expression of the *Mirn15a/16-1* is an important molecular lesion in CLL (Raveche, *et al* 2007). Future studies could explore the various mouse models of CLL to ascertain the role of environmental triggers in the augmentation of a pre-existing propensity to develop CLL.

The Role Of MicroRNAs in B Cell Lymphoproliferation/CLL

We have established that a point mutation in the 3' flanking sequence of *Mirn16-1* results in decreased levels of miR-16 in NZB. In addition, we have recently discovered that our LNC cell line has high levels of *Mirn155* expression (data not shown). Based on these facts, we have suggested a model for the role of microRNAs in B cell lymphoproliferation and CLL. B stem cells from NZB mice undergo increased lymphopoiesis, becoming self-reactive naïve B-1 cells. As a potential negative regulator of *Bcl2*, decreased levels of miR-16 may play a role in upregulation of *Bcl2* expression observed in NZB malignant B cells, (Figure 4) which protects these poly-reactive B-1 cells from undergoing apoptosis. In addition, these cells overexpress *Mirn155*, a negative regulator of activation-induced cytidine deaminase (*Aicda*). The gene product, aid, has been shown to be involved in immunoglobulin class switch recombination, and somatic hypermutation, and a requirement for aid in several crucial steps of B cell terminal differentiation has been demonstrated (Muramatsu, *et al* 2000,Revy, *et al* 2000). Therefore, overexpression of *Mirn155* may result in decreased levels of aid and be responsible for the development of pre-malignant B-1 cells with chromosomal instability. These cells also display a DNA damage repair defect and mature into malignant B-1 cells, resulting in CLL. The interaction of several pathways leading toward malignant B cell development is included in a model of NZB disease (Figure 5).

Future Directions in Mouse Models of Cancer

This report has described several mouse models of CLL and focused on the *de novo* NZB mouse model. The NZB strain demonstrates a predictable progression from early B cell hyperactivity, including hyper-IgM and autoantibody production, that continues to a late-onset B cell malignancy. The NZB strain serves as a murine model of human CLL and has been deposited in the NCI Cancer Models Database (http://cancermodels.nci.nih.gov/mmhcc) as such. The finding that NZB B cell lymphoproliferative disease is linked to a region on mouse chromosome 14, which is analogous to human 13q14 (region frequently involved in CLL), strengthens the rationale for using the NZB as a model for human CLL.

It is important to realize the following four basic differences between the mouse and human regarding haematological malignancy. The first is that the mouse bone marrow is rarely involved and is not the primary site of morphological involvement. As corollary, the mouse bone marrow is normally markedly hypercellular, which is in contrast to physiological hypocellularity in humans. The second point is that, in the mouse, the spleen is not only the site of primary involvement but it is used to classify disease instead of the lymph node, which is used in humans. Third, the murine spleen always has some level of extramedullary hematopoiesis and, in the NZB, this is often markedly increased due to the underlying autoimmune hemolytic anemia. Finally, the lymphoproliferative disease classification is not entirely parallel between the two species (Morse, *et al* 2002). That said, each of the six models discussed in this paper offer unique advantages and disadvantages. All of the models discussed (with the exception of the Eμ-mmu-miR-155 transgenic mouse model) have an

expansion of IgM^+ B cells that are dull for the expression of the CD45 isoform found on B cells, B220, as well as other markers characteristic of B-1 cells, including low level CD5 expression. This is similar to the phenotype of the malignant B cells in human CLL (Yu, *et al* 2000).

Transgenic mouse models are invaluable tools in determining the involvement of particular genes or pathways in the pathogenesis of a malignancy. However, in certain models, as is the case in the *MIRN155* transgenic mouse model, overexpression of a gene in mice that has been found to be overexpressed in human CLL resulted a phenotype that did not closely resemble the human disease. Rather, the *MIRN155* transgenic mice may be a model for polyclonal B cell expansion which in humans may or may not precede monoclonal B lymphocytosis (MBL) (Shim, *et al* 2007). In the *TNFSF13* transgenic mouse model, the onset of disease is not observed in the majority of the mice, suggesting that overexpression of *TNFSF13* alone is insufficient in inducing the malignant transformation of $CD5^+$ B cells. It is possible that crossing *TNFSF13* transgenic mice with mice overexpressing other target genes involved in CLL could produce a model with a phenotype more closely resembling human CLL. This is the case in *TRAF2DN/Bcl-2* mice. In this latter double transgenic, the disease more closely resembles CLL than either of the single gene transgenic mice. The drawback to this would be that, without a complete understanding of the interaction of all overexpressed genes, the models would not be entirely useful in testing potential therapies. The (NZB×NZW)F1 strain overexpressing the cytokine IL-5 gives insight into the role of cytokines and self reactivity in the development of B-1 cell expansions. Among the transgenic models, only the *TCL1A* transgenic mouse has been utilized for testing possible therapeutics for CLL (Zanesi, *et al* 2006). *TCL1A* has been shown to be elevated in CLL, but this may be secondary to abnormalities in the microRNA, miR-29, which affects genes in addition to *TCL1A.* The *TCL1A* model of CLL is somewhat artificial as *TCL1A* is involved in T cell leukemias and the CLL-like disease occurs only when *TCL1A* is expressed with a B cell specific promoter.

The NZB mouse strain is a *de novo* autochthonous cancer model and has many advantages over other models as metastasis is lymphatic and vascular. Autochthonous cancer models have inherent variability in the time and frequency of disease, and the time frame of study for the development of CLL in NZB mice is 9–12 months. However, the NZB has the advantage of an alternative system, the transplantable syngeneic system, in which an NZB-derived B cell malignant cell line is employed and disease is observed in weeks. Alternatively, spleen cells from an aged NZB can be injected into younger mice to study the transfer of disease.

Using the NZB mouse as a model of human CLL, insights into the human disease may be obtained. Several abnormalities in NZB result in CLL development and these include: (1) alterations in microRNA levels result in abnormal gene expression. In particular, abnormalities in *Mirn16-1* (in terms of the presence of a mutation resulting in decreased lymphocyte expression of miR-16) play a potential role in the failure to undergo apoptosis. Increased *Mirn155* expression may play a role in increased B lymphopoiesis. (2) Abnormal DNA repair found in the NZB strain may be important as a fundamental predisposition to cancer and increase the tumorgenic potential of environmental triggers, such as radiation, chemical or biological exposure. (3) The increase in telomerase activity, coupled with abnormal microRNA expression, hyper-responsiveness to self-antigen, a DNA repair defect and chromosomal instability, together contribute to the development of CLL. From this model, it is evident that CLL development is a multi-gene process with environmental triggers playing a role.

While all of these mouse models discussed above display advantages, the *de novo* NZB mouse model may present as the best model at this time. Although this model has the complexity of CLL, the ability to undertake longitudinal studies, genetically manipulate, and perform multiple assays will help uncover biochemical pathways that result from genetic alterations involved in the development of CLL.

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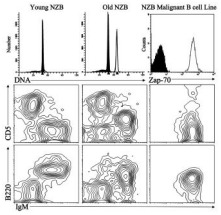

Figure 1. Flow analysis of NZB mice

Flow cytometric analysis was performed on spleens from 3-month-old (young, left panel) and 15-month-old NZB mice (middle panel) as well as an NZB-derived malignant B cell line (right panel). Single parameter staining for DNA content via propidium iodide revealed the presence of hyperdiploid cells in the spleens of old NZB mice (top middle) that were not present in young NZB (top left). Surface staining for IgM, CD5, and B220 revealed the presence of IgM^+ $CD5^+$ (middle row) and IgM^+ $B220^{dull}$ cells (bottom row) in old but not young mice. The phenotype of these cells resembles that of our isolated NZB malignant B cell line. In addition, flow cytometry shows that this B cell line expresses zap-70 (top right, filled histogram isotype control, unfilled histogram anti-zap-70), a characteristic of malignant cells in CLL.

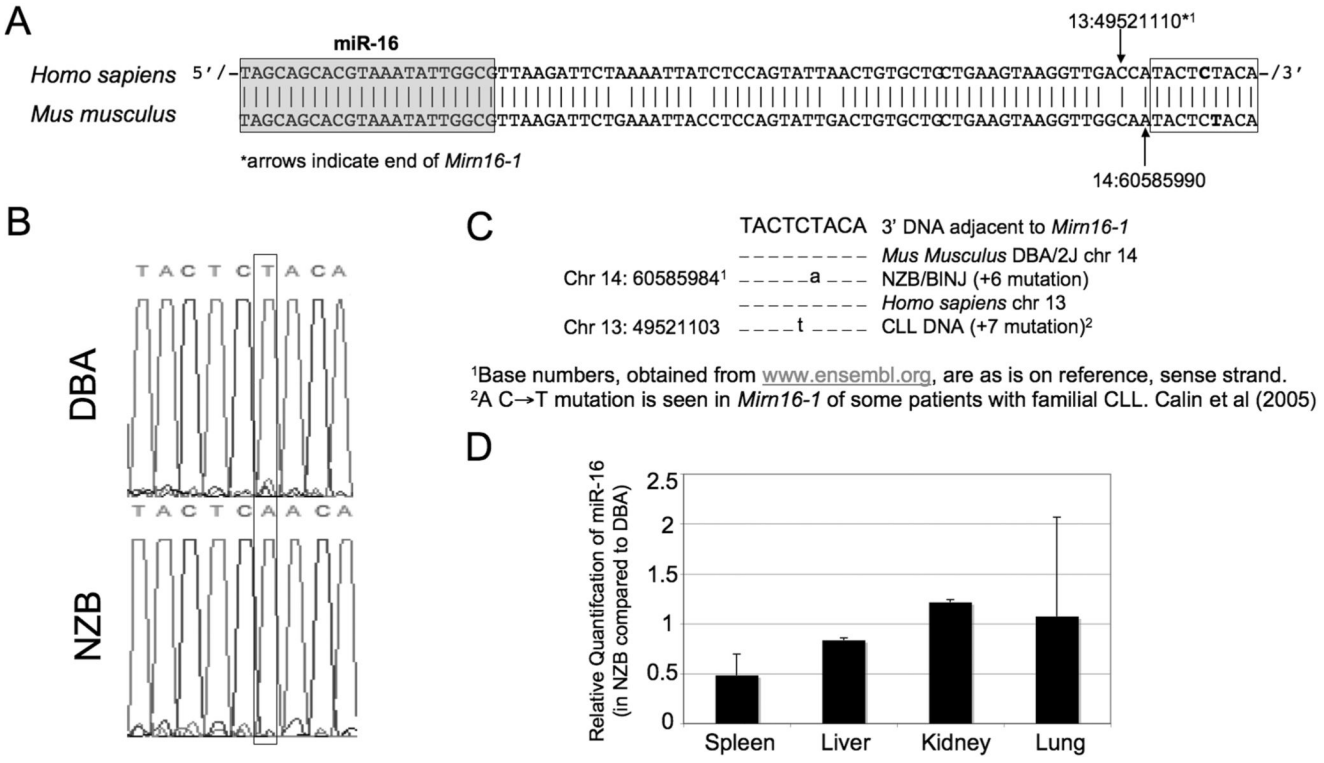

Figure 2. Point mutation in 3' DNA adjacent to Mirn16-1 region in NZB

A. Nucleotide sequence comparison of the region of mouse chromosome (chr) 14 and human chr 13 on which miRNA *MIRN16-1* is located. The top sequence is the database reference sequence (antisense strand) in *Homo sapiens* for *MIRN16-1* with sense strand base coordinates (NCBI36)13:49521099-49521187. The second sequence is the antisense of the database reference sequence in *Mus musculus* for *Mirn16-1* with the sense strand base coordinates (NCBIM36)14:60585981-60586067. The sequence homology between miR-16 (shaded box) and the *MIRN16-1* 3' flanking region (unshaded box) of *Mus musculus* (chr 14) and of *Homo sapiens* (chr 13) is shown (vertical arrows indicate the end of the *MIRN16-1* in humans versus mouse). The mutation sites in the *MIRN16-1* 3' flanking region for both human and mouse are shown in bold letters. The precursor stem-loop structure sequences (pre-miRNA) are based on established nomenclature. *B.* Chromatogram comparing the sequence of control mouse strain DBA/2J versus NZB/BlNJ. Sequences are identical to the reference sequence, except for a T→A point mutation (on the antisense strand; A →T point mutation on the sense strand of chr 14) in the NZB/BlNJ. In addition, DNA extracted from DBA/2J (5weeks) liver, NZW (5weeks) spleen, Balb/c B cell lymphoma cell line (CH27), C57BL/6 (4months) kidney, SJL/J B cell lymphoma line (NJ117), and NOD SCID (7months) liver showed no point mutation, whereas DNA from NZB/BlNJ spleen, liver (14months), kidney (15months), T cell lymphoma line (3C2), and malignant B1 cell line (LNC) all had the same point mutation (data not shown). In the mouse samples, both *Mirn15a* (Accession # MI0000564) and *Mirn16-1* (MI0000565) and mature sequences for both miR-15a (MIMAT0000526) and miR-16 (MIMAT0000527) were also compared and no other mutation was found in these regions (data not shown). *C* Sequence comparison between human and mouse 3' adjacent to *MIRN16-1*. The point mutations in NZB/BlNJ splenic DNA and in the reported DNA of patients with CLL are indicated (Calin et al 2005). The NZB/BlNJ splenic DNA shows an A->T mutation at the 60585984 base on chromosome 14, 6 bases from the end of the *Mirn16-1* sequence. CLL DNA has a reported G->A mutation at the 49521103 base on chr 13, 7 bases from the

MIRN16-1 sequence (Calin et al 2005). *D.* Real Time PCR was used to determine the levels of miR-16 in tissues collected from NZB mice relative to DBA. The results show that NZB mice have a significant decrease in the spleen of the levels of miR-16 compared to DBA mice.

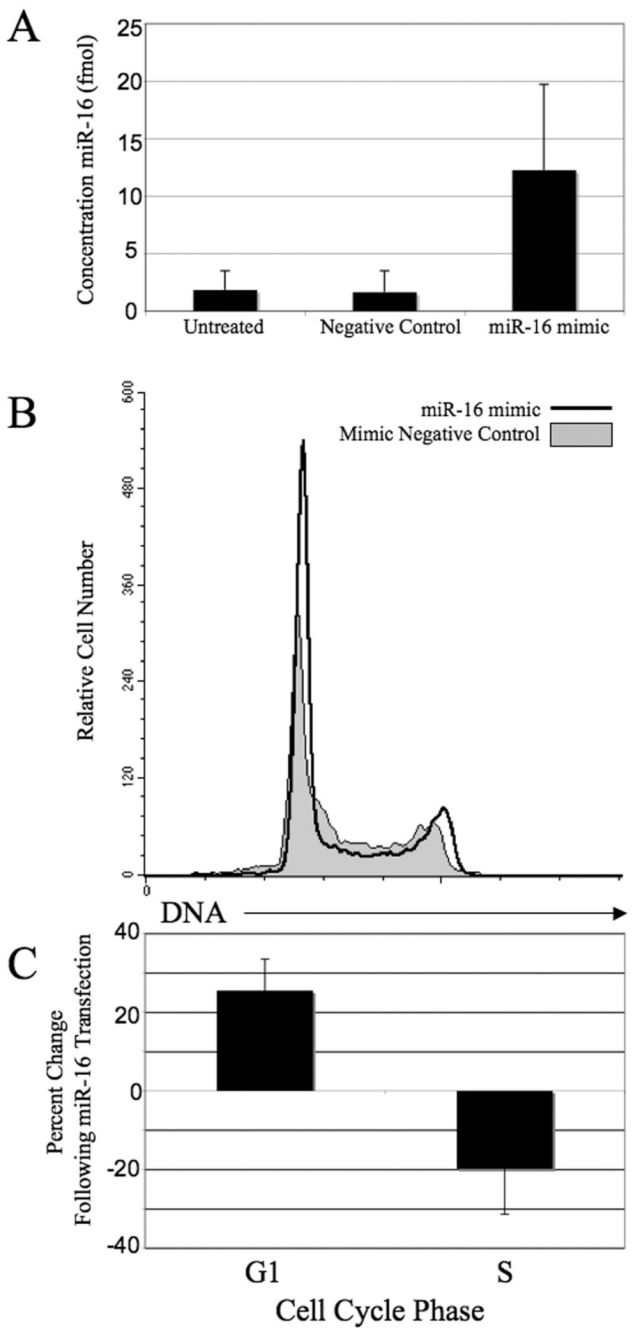

Figure 3. Transfection of miR-16 into an NZB malignant B cell line

Our NZB malignant B cell line was transfected via the Amaxa Nucleofector II with 3ug of Mouse *miRIDIAN* mmu-miR-16 Mimic or *miRIDIAN* microRNA Mimic Negative Control (Dharmacon, Lafayette, CO) using Solution T and program G-16 according to the manufacturer's instructions. *A.* Levels of the miR-16 were determined by Real-Time PCR. Transfection with the miR-16 mimic resulted in a >5-fold increase in the levels of mature miR-16 as compared to untransfected and Mimic Negative Control transfected cells. *B.* To determine the effects of miR-16 on the cell cycle, cells were stained with propidium iodide 24 h post-transfection and analyzed for DNA content with Modfit LT software (Verity Software House). *C.* Analysis with Modfit LT software showed a decrease in the percentage

of cells in S phase coupled with a G1 arrest following transfection with the miR-16 mimic compared to the negative control.

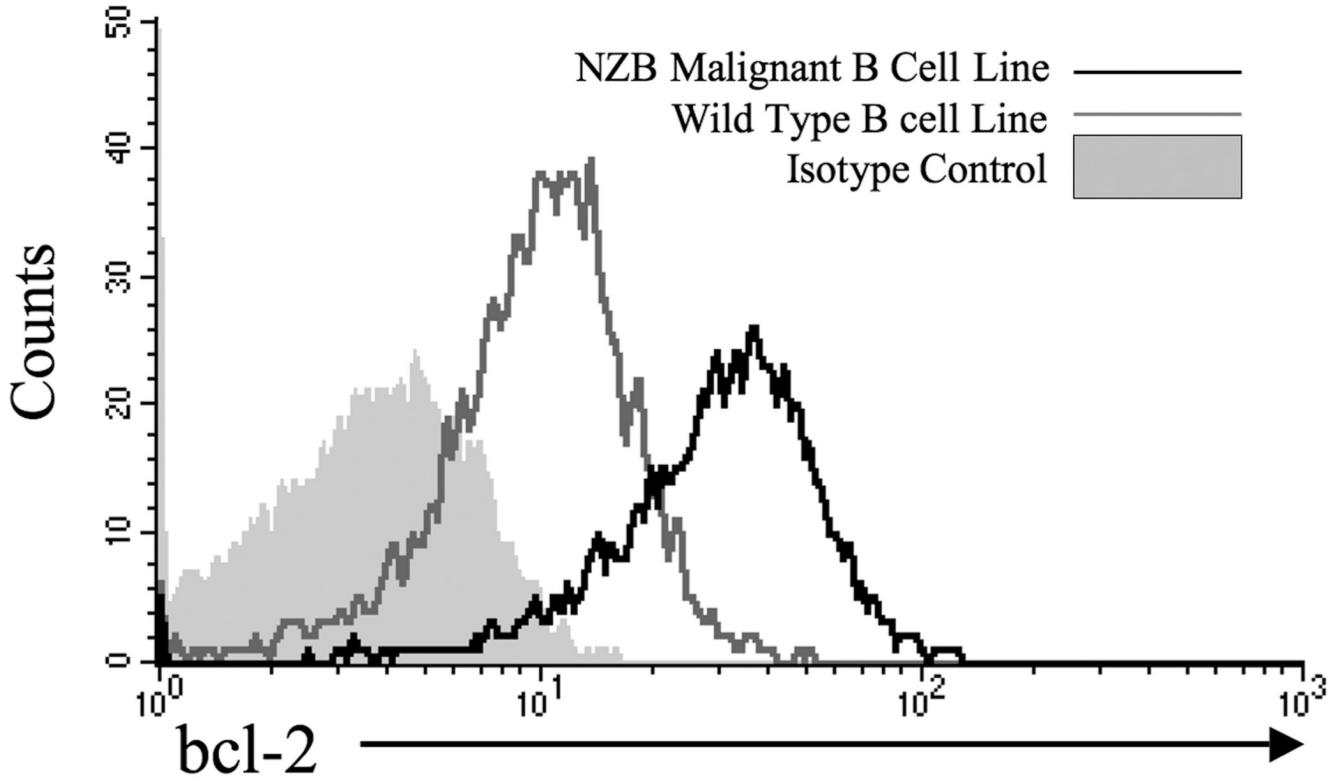

Figure 4. Elevated levels of bcl-2 in Malignant B cells from NZB mice

Flow cytometry was employed to evaluate the levels of bcl-2 in our NZB malignant B-1 cell line (LNC) as compared to a wild type control cell line (A20). Cells were stained with a phycoerythrin-conjugated anti-mouse bcl-2 antibody (Pharmingen San Diego, CA), acquired on a FACSCalibur (Becton Dickinson, San Jose, CA) and analyzed with CellQuest Software (Becton Dickinson, San Jose, CA). We found that LNC (black line) has increased expression of *Bcl2* when compared to A20. The shaded region represents the isotype control.

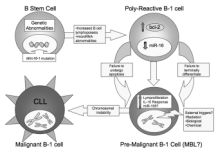

Figure 5. A model of the development of CLL in NZB mice

A point mutation in the 3' flanking sequence of the precursor microRNA, *Mirn16-1* is seen in all cells of NZB, including stem cells. Several possible genetic defects (including the *Mirn16-1* mutation) may lead to increased B lymphopoiesis. The developing B-1 cells are polyreactive (including autoreactivity) and express decreased levels of miR-16. These cells are protected from apoptosis by increased levels of bcl-2. In addition to increased response to IL-10, these cells express elevated levels of miR-155, which may downregulate activation-induced cytidine deaminase (*Aicda*), perhaps preventing B cell terminal differentiation. Pre-malignant cells develop (at the monoclonal B lymphocytosis or MBL stage) and may undergo further alterations perhaps due to external triggers, including radiation, chemical or biological. The underlying DNA repair defects may lead to chromosomal instability, further leading to the development of a malignant B-1 clone, resulting in CLL.

Table 1

Murine Models of CLL

Background Strain (References)	Transgene	Phenotype of Expanded B Cells	Earliest Disease Manifestations	Distinguishing Characteristics
C57Bl/6 and FVB/N (Costinean *et al* 2006)	*MIRN155*	$IgM^{-}B220^{dull}$ $CD5^{-}$ TCR^{-} $CD43^{-}$	Splenomegaly at 3 weeks of age	Expanded cells polyclonal with no chromosomal abnormalities (not CLL)
BALB/c X FVB//N (Zapata *et al* 2004)	*TRAFDN /Bcl2*	IgM^{+} $B220^{dull}$ $CD5^{dull}$ $CD11b^{dull}$	Splenomegaly	Clonal B-1 cells. Death as early as 6mo, 20% survival rate by 14mo
B6C3 (Bichi *et al* 2002)	*TCL1A*	IgM^{+} $B220^{dull}$ $CD5^{+}CD11b^{dull}$	Expansion of B1 cells in peritoneum at 2 months	Development of secondary malignancies in 20% of mice
C57Bl/6 (Planelles *et al* 2004)	*TNFSF13* (*APRIL*)	IgM^{+} $B220^{dull}$ $CD5^{dull}$	Expansion of B1 cells in peritoneum at 4 months	Mild form of disease with no effect on life-span
(NZB X NZW)F1 (Wen *et al* 2004)	*IL5*	IgM^{+} $B220^{dull}$ $CD5^{dull}$	Higher frequency of B-1 cells by 5 months	Increased survival and reduction of underlying autoimmune disease
NZB (Raveche *et al* 2007)	*de novo*	IgM^{+} $B220^{dull}$ $CD5^{dull}$	Expansion of B-1 cells in spleen and peritoneum at 4 months	*Mirn16-1* loci mutation, decreased miR-16 in spleen, chromosomal abnormalities. Clonal B-1 cells