IRF4 Is a Suppressor of c-Myc Induced B Cell Leukemia

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Abstract

Interferon regulatory factor 4 (IRF4) is a critical transcriptional regulator in B cell development and function. We have previously shown that IRF4, together with IRF8, orchestrates pre-B cell development by limiting pre-B cell expansion and by promoting pre-B cell differentiation. Here, we report that IRF4 suppresses c-Myc induced leukemia in EμMyc mice. Our results show that c-Myc induced leukemia was greatly accelerated in the IRF4 heterozygous mice (IRF4+/−/−Myc); the average age of mortality in the IRF4+/−/−Myc mice was only 7 to 8 weeks but was 20 weeks in the control mice. Our results show that IRF4+/−/−Myc leukemic cells were derived from large pre-B cells and were hyperproliferative and resistant to apoptosis. Further analysis revealed that the majority of IRF4+/−/−Myc leukemic cells inactivated the wild-type IRF4 allele and contained defects in Arf-p53 tumor suppressor pathway. p27kip is part of the molecular circuitry that controls pre-B cell expansion. Our results show that expression of p27kip was lost in the IRF4+/−/−Myc leukemic cells and reconstitution of IRF4 expression in those cells induced p27kip and inhibited their expansion. Thus, IRF4 functions as a classical tumor suppressor to inhibit c-Myc induced B cell leukemia in EμMyc mice.

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Introduction

B cell development features a sequential rearrangement of immunoglobulin heavy and light chain loci and expression of distinct cell surface markers [1]. After productive heavy chain rearrangement at the pro-B stage, the newly synthesized heavy chain pairs with surrogate light chains and forms the pre-B cell receptor (pre-BCR). Pre-B cells consist of two distinct subsets: large pre-B and small pre-B cells. Large pre-B cells are cycling cells expressing pre-BCR whereas small pre-B cells are quiescent cells following cell cycle exit. Pre-B cells expansion and the subsequent transition from large pre-B to small pre-B cells are tightly regulated during B cell development and is dependent on signals from the pre-BCR and IL-7 receptor [2]. Disruption of this coordinated developmental process can lead to abnormal B cells development and transformation. Indeed, acute lymphoblastic leukemia (ALL) is often derived from pre-B cells that exhibit defects in proliferation and differentiation [3].

Interferon regulatory factor 4 (IRF4), is expressed predominantly in the immune system and plays an important role in its development and function [4]. IRF4, together with IRF8, are critical for the pre-B cell development. In the absence of IRF4 and IRF8, B cell development is blocked at the large pre-B stage [5]. We have shown that IRF4 limits pre-B cell expansion by inducing Ikaros and Aiolos which in turn directly suppress c-Myc expression [6,7]. In addition, IRF4 is critical for light chain rearrangement and receptor editing [5,9,10]. Beside its role at the pre-B stage, IRF4 is also required for mature B cell function. It has been shown that mice lacking IRF4 (IRF4−−/−) fail to generate plasma cells and are defective in response to T cell dependent and independent antigens [11]. Recent studies have further shown that IRF4 is critical for the class-switch recombination by inducing activation induced deaminase (AID) and for germinal center reaction by downregulating Bcl6 [12,13,14]. IRF4 has been found to induce c-Myc expression in multiple myeloma cells and is critical for their survival and expansion [15]. Finally, IRF4 can induce the expression of Fas apoptosis inhibitory molecule (FAIM) to regulate mature B cell survival and apoptosis [16].

Given its role as a critical transcriptional regulator that limits pre-B cell expansion and promotes pre-B cell differentiation, it is reasonable to assume that IRF4 may function as a tumor suppressor against pre-B cell transformation. Indeed, a previous study has shown that IRF4 functions as a tumor suppressor to inhibit BCR/ABL oncogene induced B cell acute lymphoblastic leukemia (B-ALL) [17]. In addition, mice deficient for both IRF4 and IRF8 develop lymphoblastic leukemia [18]. Although IRF4 can suppress BCR/ABL induced B cell transformation, the molecular mechanism by which IRF4 exerts its function remains unclear. In this report, we assessed the role of IRF4 in c-Myc oncogene induced B cell transformation by breeding IRF4 deficient mice with EμMyc transgenic mice. In the EμMyc mice, the expression of c-Myc oncogene is driven by immunoglobulin heavy chain enhancers and is predominantly found in the B cells. EμMyc transgenic mice mainly develop two types of leukemia/lymphoma: pro/pre-B derived and mature B cell derived and the majority of the EμMyc mice succumb to disease within 5 to 6 months of age [19]. It has been shown that the leukemogenesis of...
EqMyc mice can be modulated by oncogenes and tumor suppressor genes and thus, EqMyc mice have been widely used as an animal model to assess the role of potential oncogenes or tumor suppressor genes in B cell transformation [20,21,22,23].

In this report, we show that c-Myc induced leukemia was greatly accelerated in the IRF4 heterozygous mutant mice. Moreover, we provided evidence that IRF4 functions as a classical tumor suppressor gene to inhibit c-Myc induced leukemogenesis. Our results further revealed that deficiency of IRF4 accelerated the loss of p27kip in the EqMyc mice and reconstitution of IRF4 expression in leukemic cells restored p27kip expression in leukemic cells and inhibited their proliferation in vivo.

Results

C-Myc induced leukemia was accelerated in the IRF4−/− M yc mice

We did not observe a significant increase in tumor formation in IRF4 deficient mice, indicating that deficiency for IRF4 alone isn’t sufficient for tumor development. Here, we wanted to examine whether deficiency of IRF4 synergizes with the c-Myc oncogene to induce B cell leukemia and lymphoma. We crossed IRF4−/− mice with the EqMyc mice and generated IRF4 heterozygous mutant mice expressing EqMyc transgene (IRF4+/−/Myc). To our surprise, IRF4+/−/Myc mice showed a dramatically accelerated mortality with a median age of 7 to 8 weeks (Fig. 1A). In contrast, the median age of mortality for IRF4+/+/Myc mice is 20 weeks (Fig. 1A). By five weeks of age, IRF4+/−/Myc mice had already massively enlarged spleens, while this was not evident in the IRF4+/+/Myc mice of the same age (Figure S1A). H&E staining of isolated spleens further shows that while the demarcation of white and red pulps is clearly discernible in IRF4+/− and IRF4+/−/Myc mice, it disappeared in the IRF4+/−/Myc mice due to the expansion and infiltration of leukemic cells.

FACS analysis of cells in the bone marrow and spleen further show that there was a massive expansion of B220+ B cells in the IRF4+/−/Myc mice (Fig. 1C/D and Tab. 1/2). Compared to IRF4+/+/Myc mice, the absolute numbers of B220+ B cells was found to be 3-fold higher in bone marrow and 10-fold higher in spleen. While the absolute numbers of B220+IgM+ B cells in bone marrow and spleen were comparable between IRF4+/−/Myc and IRF4+/−/Myc mice, the absolute numbers of B220+IgM− B cells were dramatically increased in the IRF4+/−/Myc mice (Fig. 1C/D and Tab 1/2). The massive expansion of leukemic cells also severely affected the development of other blood lineages. As shown in Fig. 1C, the percentages of CD11b+ myeloid cells in bone marrow decreased from 32% in the IRF4+/−/Myc mice to only 1% in the IRF4+/−/Myc mice. In spleen, the percentage of Thy1+ T cells decreased from 21% in IRF4+/−/Myc mice to only 3% in the IRF4+/−/Myc mice. The expansion and infiltration of leukemic cells also caused severe erosion and thinning of the femur and tibia. In addition, the B cell population (mainly B220+IgM−) was also increased in the blood of IRF4+/−/Myc mice (Fig. 1E and data not shown). The leukemic cells also infiltrated the lymph node, Thymus and liver, resulting in mild to moderate enlargement of those organs. Two additional sets of independent analysis can be found in Figure S1. Collectively, our results indicate that the IRF4+/−/Myc mice developed leukemia that originated from B cell progenitors.

IRF4+/−/Myc leukemic cells were derived from large pre-B cells and were transplantable

To determine the identity of B220+IgM− cells in the IRF4+/−/Myc mice, we stained the cells with a panel of cell surface as well as intracellular markers such as CD43, Bp-1, intracellular mu and surrogate light chain λ5. Pre-B cells express Bp-1 but do not express CD43. The heavy chain protein μ (μ) can be detected intracellularly in the pre-B but not the pro-B cells. λ5 is a component of pre-B cell receptor found only on the cell surface of pre-B cells. FACS analysis showed that B220+IgM− leukemic cells in the IRF4+/−/Myc mice did not express CD43 but express a high level of Bp-1, intracellular μ and surrogate light chain λ5 (Fig. 2A/B/C/D). Collectively, these results indicate that the B220+IgM− leukemic cells were derived from the large pre-B cells. To determine if the leukemic cells derived from IRF4+/−/Myc mice are transplantable, we injected IRF4+/−/Myc bone marrow into non-irradiated wild type syngenic host mice. All ten recipient mice developed leukemia similar to IRF4+/−/Myc mice (Fig. 2E) and died within two month of transplantation. Two additional sets of independent analysis can be found in Figure S2. In summary, IRF4+/−/Myc mice develop pre-B cells derived leukemia that is transplantable in the syngenic mice.

The B220+IgM− cells in IRF4+/−/Myc mice exhibited enhanced proliferation and reduced apoptosis

We wanted to identify the molecular defects that led to the accelerated leukemogenesis in the IRF4+/−/Myc mice. To this end, we examined the survival and proliferation of B220+IgM− cells in the bone marrow of 4-weeks old IRF4+/−/Myc mice prior to the development of symptoms. At this age, although there was expansion of B220+IgM− cells in the bone marrow and spleen of IRF4+/−/Myc mice, there were few B220+IgM− cells in the blood (data not shown). Bone marrow cells were isolated from IRF4+/−, IRF4+/−/Myc and IRF4+/−/Myc mice. After surface staining, the cell cycle status of B220+IgM− cells was examined with Hoechst dye as previously described [6,7]. Compared to the IRF4+/−/Myc mice, B220+IgM− cells in the IRF4+/−/Myc mice were hyperproliferative as 38±8% of B220+IgM− cells were cycling (in S and G2/M phases) in the IRF4+/−/Myc mice compared to 30±6% in the IRF4+/−/Myc mice (Fig. 3A). BrdU pulse-labeling analysis further revealed that the B220+IgM− cells in IRF4+/−/Myc mice were cycling faster than their counterparts in the IRF4+/−/Myc mice (data not shown). TUNEL analysis further revealed that 4.8±1.1% of B220+IgM− cells in the IRF4+/−/Myc mice were apoptotic whereas only 1.2±0.6% of them in the IRF4+/−/Myc mice underwent apoptosis (Fig. 3B). Collectively, these results show that the expanded B220+IgM− cells in the IRF4+/−/Myc mice are hyperproliferative and resistant to apoptosis.

Loss of the expression of p27kip and disruption of the Arf-p53 pathway in the IRF4+/−/Myc leukemic cells

P27kip is a cell cycle inhibitor, and loss of p27kip expression has been shown to accelerate c-Myc induced leukemia and lymphoma [23]. Moreover, our previous study has shown that p27kip is part of molecular circuitry that is responsible for shutting down pre-B cell proliferation [6]. Here, we further examined the expression of p27kip in the B220+IgM− cells described in Fig. 3A. Although p27kip expression was readily detectable in all three IRF4+/−/Myc cells, its expression was lost in all six IRF4+/−/Myc cells (Fig. 4A). Importantly, expression of c-Myc was comparable between IRF4+/−/Myc and IRF4+/−/Myc cells (Fig. 4A). To further assess the role of p27kip in the proliferation of IRF4+/−/Myc leukemic cells, we transduced cultured IRF4+/−/Myc leukemic cells with p27kip expression plasmid. As shown in Fig. 4B, restoring the expression of p27kip dramatically inhibited the proliferation of IRF4+/−/Myc leukemic cells. The percentage of cycling cells decreased from 35% in the control to 12% in the p27kip transduced cells. Collectively, these results suggest that defective expression of p27kip contributes to the hyperproliferative index of IRF4+/−/Myc leukemic cells.
The development of leukemia and lymphoma in EμMyc mice is dependent on the disruption of Arf-p53 tumor suppressor pathway [24,25]. It has been shown that p53 is frequently mutated in EμMyc tumor [24]. The mutant form of p53 is often overexpressed and functions as a dominant negative mutant. To determine if Arf-p53 pathway is disrupted in the IRF4+/2 Myc leukemic cells, we measured the expression of p53, Arf and c-Myc in IRF4+/2 leukemic cells isolated from twelve different diseased mice. Our results show that p53 was overexpressed in three IRF4+/2 Myc leukemic cells (Fig. 4C). Further sequence analysis shows that all three cells contained a p53 misense mutation R270C, which corresponds with a mutation hotspot of p53 found in human tumors [26]. Expression of Arf is transcriptionally suppressed by p53 and the loss of p53 function often lead to overexpression of Arf in EμMyc tumor cells. Indeed, Arf was overexpressed in IRF4+/2 Myc leukemic cells expressing mutant p53 (Fig. 4C). Overall, three out of twelve IRF4+/2 Myc clones expressed mutant p53, a frequency that is similar to what has been reported in wild type EμMyc mice [24].

IRF4 functions as a tumor suppressor in c-Myc induced leukemia

The finding that c-Myc induced leukemia was dramatically accelerated in the IRF4+/2 Myc mice indicates that IRF4 functions as a tumor suppressor in c-Myc induced leukemia. During tumorigen-
IRF4 Suppresses c-Myc Induced Leukemia

Discussion

In this report, we provide evidence that IRF4 functions as a tumor suppressor in c-Myc induced B cell leukemia. Our results show that c-Myc induced leukemia was dramatically accelerated in IRF4+/− Mice. Moreover, five out of six IRF4+/− Mice leukemic clones further inactivated the remaining wild type IRF4 allele, resulting in a complete loss of IRF4 expression in those cells. Our finding is consistent with a previous study which shows that IRF4 functions as tumor suppressor in BCR/ABL oncogene induced B-ALL and further demonstrates that IRF4 is capable of functioning as a tumor suppressor against a broad spectrum of oncogene insults at the pre-B stage. The accelerated leukemogenesis in the IRF4+/− Mice can be caused by defect in pre-B cell development. Progenitor B cells are expanded in wild type EμMyc mice. As IRF4 is a critical regulator of pre-B cell differentiation, loss of IRF4 expression can further exacerbate the defect in early B cell development in the EμMyc mice, causing further expansion of progenitor B cells pool that could serve as targets for subsequent transformation.

C-Myc induced leukemia and lymphoma is held in check by p27kip, which inhibit cell cycle progression and by the Arf/p53 pathway that promotes apoptosis [20,24,32]. However, the frequency of p53 mutation in the IRF4+/− Mice leukemic cells is similar to wild type EμMyc cells, suggesting that accelerated leukemogenesis in IRF4+/− Mice mice isn’t a result of high frequency of p53 mutation. Instead, our results indicate that deficiency of IRF4 accelerates the loss of p27kip in c-Myc overexpressing cells. First, the expanded B220+IgM− cells in IRF4+/− Mice mice lost the expression of p27kip and were hyperproliferative; second, reconstitution of IRF4 expression in IRF4+/− Mice leukemic cells induced the expression of p27kip and

Table 1. Absolute number of B220+, B220+IgM− and B220+IgM+ B cells in bone marrow of IRF4+/+, IRF4+/−, IRF4+/+Myc and IRF4+/−Myc mice (×10^6).

| Genotype       | B220+ | B220+IgM− | B220+IgM+ |
|----------------|-------|-----------|-----------|
| IRF4+/+        | 3.8±2.1 | 2.5±1.4   | 1.3±0.6   |
| IRF4+/−        | 3.1±0.7 | 1.9±0.6   | 1.0±0.2   |
| IRF4+/+Myc     | 6.1±2.3 | 5.1±2.7   | 1.0±0.1   |
| IRF4+/−Myc     | 19.5±6.4** | 18.0±5.7** | 1.3±0.4   |

Total number of B220+, B220+IgM− and B220+IgM+ B cells in mice of different genotypes under study. Cell were isolated from spleen of five-week-old IRF4+/+, IRF4+/−, IRF4+/+Myc and IRF4+/−Myc mice. The cells were stained with antibodies against B220 and IgM and analyzed by FACS. The numbers are the averages and standard deviations of the results for a total of five mice in each group.

**p<0.01 (compared to their counterparts in the IRF4+/+Myc mice).

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Table 2. Absolute number of B220+, B220+IgM− and B220+IgM+ B cells in spleen of IRF4+/+, IRF4+/−, IRF4+/+Myc and IRF4+/−Myc mice (×10^6).

| Genotype       | B220+ | B220+IgM− | B220+IgM+ |
|----------------|-------|-----------|-----------|
| IRF4+/+        | 29.7±13.5 | 0.9±0.4   | 29.6±12.5 |
| IRF4+/−        | 15.0±6.2 | 0.5±0.4   | 14.7±5.8   |
| IRF4+/+Myc     | 14.7±7.8 | 0.6±0.3   | 14.3±8.0   |
| IRF4+/−Myc     | 141.0±48.0** | 128.0±40.4** | 12.7±7.8 |

Total number of B220+, B220+IgM− and B220+IgM+ B cells in mice of different genotypes under study. Cell were isolated from spleen of five-week-old IRF4+/+, IRF4+/−, IRF4+/+Myc and IRF4+/−Myc mice. The cells were stained with antibodies against B220 and IgM and analyzed by FACS. The numbers are the averages and standard deviations of the results for a total of five mice in each group.

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To further examine the effect of IRF4 on c-Myc induced leukemogenesis, we developed a doxycycline (Dox) inducible, bitransgenic mouse model system in which the expression of IRF4 can be regulated in vivo by Dox. The first transgenic line MMTV-tTA was developed by Hennighausen et al, in which the expression of the tetracycline transactivator gene (tTA) is driven by the mouse mammary tumor virus LTR [27]. In this system, the tTA is active in the absence but not in the presence of Dox. Interestingly, it has been demonstrated that the tTA is expressed at a high level in the B cells of MMTV-tTA mice and thus, this model has been successfully used to induce B cell specific transgene expression [28,29,30,31]. As shown in Fig. 5B, expression of the IRF4 transgene is driven by a TRE promoter which consists of a minimal CMV promoter linked to tetracycline response elements.

In contrast, the expression of c-Myc was not significantly affected in the IRF4+/− Myc leukemic cells.

Discussion

In this report, we provide evidence that IRF4 functions as a tumor suppressor in c-Myc induced B cell leukemia. Our results show that c-Myc induced leukemia was dramatically accelerated in IRF4+/− Mice mice. Moreover, five out of six IRF4+/− Mice leukemic clones further inactivated the remaining wild type IRF4 allele, resulting in a complete loss of IRF4 expression in those cells. Our finding is consistent with a previous study which shows that IRF4 functions as tumor suppressor in BCR/ABL oncogene induced B-ALL and further demonstrates that IRF4 is capable of functioning as a tumor suppressor against a broad spectrum of oncogene insults at the pre-B stage. The accelerated leukemogenesis in the IRF4+/− Mice mice can be caused by defect in pre-B cell development. Progenitor B cells are expanded in wild type EμMyc mice. As IRF4 is a critical regulator of pre-B cell differentiation, loss of IRF4 expression can further exacerbate the defect in early B cell development in the EμMyc mice, causing further expansion of progenitor B cells pool that could serve as targets for subsequent transformation.

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inhibited their proliferation. It has been shown that Ikaros can induce the expression of p27kip in leukemic cells [33]. As expression of Ikaros is induced by IRF4, it is possible that the induction of p27kip expression could be mediated by Ikaros. It is also possible that loss of IRF4 expression somehow accelerates the loss of p27kip in c-Myc overexpressing cells through mechanisms independent of Ikaros.

Pre-B cell receptor signaling has been proposed as a guardian of pre-B cell transformation by coordinating pre-B cell expansion and differentiation. IRF4 expression is dependent on pre-BCR signaling which is transduced by downstream signaling molecules such as Btk, Bnk and PLCγ2. Moreover, previous studies have identified Btk, Bnk and PLCγ2 as potential tumor suppressors against pre-B cell transformation [34,35,36,37]. Interestingly, a previous study has shown that c-Myc induced leukemia/lymphoma is accelerated in PLCγ2+/−Myc mice [34]. Similar to IRF4, PLCγ2 is critical for pre-B cell development and in its absence, pre-B cell development is partially blocked, resulting in an expansion of pre-B cells. Interestingly, like IRF4+/−Myc leukemic cells, PLCγ2−/−Myc tumor cells lost the expression of p27kip and didn’t exhibit enhanced frequency of p53 mutation [34]. However, PLCγ2 does not behave like classical tumor suppressor as c-Myc induced leukemogenesis remains unaltered in the PLCγ2+/−Myc mice. In contrast, our results show that IRF4 behaves like a classical tumor suppressor, downstream of pre-BCR signaling, that functions to inhibit c-Myc induced leukemia.

**Materials and Methods**

**Mice**

IRF4 mutant mice (IRF4−/−) have been previously described [11]. EmMyc transgenic mice were purchased from Jackson Laboratories [19]. EmMyc transgenic mice were bred with IRF4−/− mice to generate IRF4+/−Myc mice that are hemizygous for Myc transgene (IRF4+/−Myc). 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 (Permit Number: 10-015-05). The mice aged from 5 to 30 weeks were used for this study.

**Cell culture and retroviral infection.** B220+ cells were isolated from bone marrow of IRF4+/−Myc mice using a MACS
separation column (Miltenyi Biotech). Purified cells were overlaid on top of an irradiated S17 stromal cell layer in Opti-MEM (Gibco) medium containing 5% FBS, 50 μM b-mercaptoethanol, 2 mM L-glutamine, 100 U penicillin-streptomycin and 5 ng/ml IL-7 (R&D). S17 is a bone marrow stromal cell line that can support both myeloid and B lymphocyte development. Retroviral vectors expressing p27kip have been described previously [7].

Retroviral infection of cultured IRF4+/2Myc leukemic cells was conducted as described previously [7]. The infected cells were analyzed by FACS two days after infection.

Fluorescence-activated cell sorter analysis (FACS), TUNEL, and cell cycle analyses

5–10 weeks old IRF4+/+, IRF4+/2, IRF4+/2Myc and IRF4+/2Myc mice were used for FACS analysis. Cells were pre-incubated with either 2% rat serum or Fc-Block (2.4G2), and stained with optimal amounts of specific antibodies, either biotinylated or directly fluorophore-conjugated. Antibodies against B220 (RA3-6B2), CD19 (ID3), Thy-1, CD43, Bp-1, IgM and λ5 were purchased from Pharmingen; FACS analysis was performed with a FACS Calibur flow cytometer. The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was carried out with an APO-Direct Kit (Pharmingen). B cells labeled with dUTP in the absence of terminal transferase were used as negative control. The stained cells were analyzed by FACS. Cell cycle analysis with live cells was conducted using Hoechst 33342 dye as previously described [7].

Bone marrow transplantation

Bone marrow cells were isolated from hind limbs of the mice. The whole bone marrow cells were injected via retro-orbital sinus into nonirradiated syngenic host mice at 1×10⁶ cells per mice. B cell population in the recipient mice was analyzed by FACS after five to 10 weeks.

Western Blot and Immunohistochemistry analysis

Isolated B cells were lysed and used for Western blot analysis. The signals were visualized using the SuperSignal West Dura HRP Detection kit (Pierce). The information for the antibodies

Figure 3. B220+IgM− cells in IRF4+/2Myc mice exhibited enhanced proliferation and reduced apoptosis. Bone marrow cells were isolated from four-week old IRF4+/+, IRF4+/2Myc and IRF4+/2Myc mice. The cells were stained with antibodies against B220 an IgM. A) To analyze cell cycle status, the stained cells were incubated with Hoechst dye (10 μg/ml) and analyze by LSR II Flow Cytometer. The percentages of cycling cells among B220+IgM− population in different groups were shown. B) To detect apoptotic cells, the stained cells were fixed and permeabilized. The percentages of apoptotic cells among B220+IgM− population were determined with a TUNEL kit and plotted as fold changes over IRF4+/− control mice. The results are average and standard deviation of the values obtained from six independent experiments. * p<0.05; ** p<0.01. doi:10.1371/journal.pone.0022628.g003

Figure 4. loss the expression of p27kip and disruption of Arf-p53 pathway in IRF4+/2Myc leukemic cells. A) Bone marrow cells were isolated from four-week old IRF4+/2Myc (n = 3) and IRF4+/−Myc mice (n = 6). The B220+IgM− population were isolated via sorting and were lysed. Western blot analysis was carried out with antibodies against p27, Myc and β-actin. B) IRF4+/2Myc leukemic cells were cultivated in RPMI-1640 plus 5% FBS in the presence of IL7 (5 ng/ml). The cells were infected with retrovirus expressing either MigR1 (control) or MigR1-p27 (p27). Two days after infection, the infected cells were incubated with Hoechst dye and analyzed by LSR II Flow Cytometer. The percentages of cycling cells in control and p27 infected cells were shown. The results are average and standard deviation of the values obtained from three independent experiments. ** * p<0.01 C) leukemic cells were isolated from twelve sick IRF4+/−Myc mice and lysed for Western blot analysis. The antibodies against p53, Arf, Myc and β-actin were used. doi:10.1371/journal.pone.0022628.g004

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used in this study: antibodies against IRF4, p27kip1, Myc and β-actin (Santa Cruz); antibody against Arf (ab80, Abcam) and antibody against p53 (Ab7, Calbiochem). For immunohistochemical analysis, the spleens were fixed in formalin and paraffin-embedded. H&E staining was carried out at the tissue process core facility of the University of Nebraska Medical Center.

**Generation of an inducible IRF4 transgenic mouse**

To generate Doxycline (Dox) responsive IRF4 transgenic mice, a full-length IRF4 cDNA with a HA tag at the N-terminus was inserted into the p1Tet-Splice vector, generating the transgenic plasmid (IRF4Tg). The expression of the IRF4 transgene is driven by a TRE promoter which consists of a minimal CMV promoter fused with a tetracycline response element. Microinjections were performed by the Transgenic Mouse Core Facility at the University of Nebraska Medical Center. Six different founder lines were established and crossed with another transgenic line which expresses tetracycline transactivator gene MMTV-tTA mice (Jackson lab). In this system, tTA becomes active in the absence but not in the presence of Dox. After crossing, all lines were found to express IRF4 in the absence of Dox and the one expressing the highest level of IRF4 was chosen for further analysis. 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 (Permit Number: 10-015-05). The mice aged from 5 to 30 weeks were used for this study.

**Supporting Information**

**Figure S1 Two additional sets of independent experiments to show that c-Myc induced leukemia is acceler-**
ated in the IRF4/−/Myc mice. A) splenomegaly in IRF4/−/Myc mice B) Spleens were isolated from IRF4/−/−, IRF4+/−/Myc and IRF4+/+Myc mice and paraffin-embedded for H&E staining analysis. The stained tissues were examined under both low (2.5×) and high (10×) magnification. C/D/E) Cells were isolated from the bone marrow (C), spleen (D) and blood (E) of six-week old IRF4/−/−, IRF4+/−/Myc and IRF4+/+Myc mice, stained with indicated antibodies and analyzed by FACS. Numbers indicated the percentages of cells in the respective quadrant. CD11b+ myeloid cells in the bone marrow were examined under a live cell gate while B and T cells were examined under a lymphocyte gate. The data shown are representative of at least three independent experiments.

(TIF)

Figure S2 Two additional sets of independent experiments to show that IRF4+/-Myc leukemic cells were derived from large pre-B cells and were transplatable. A/B/C) Bone marrow cells were isolated from six-week old IRF4/−/−, IRF4+/−/Myc and IRF4+/+Myc mice. The cells were stained with antibodies against CD43, Bp-1, CD19 and B220. The stained cells were analyzed by FACS. Numbers are percentages of cells in the respective quadrant. A) IRF4+/+Myc leukemic cells expressed intracellular IRF4. The bone marrow cells isolated above were stained with antibodies against B220 and CD43. After fixation and permeabilization, the expression of intracellular heavy chain μ was detected with an anti-IgM antibody. The isotype IgG1 antibody staining was used as a control for non-specific binding. The dark area (control IgG1) and light area (anti-IgM). D) IRF4+/−/Myc cells expressed surrogate light chain λ5. Bone marrow cells isolated from IRF4+/−/− and IRF4+/−Myc mice were cultured in presence of IL-7 for two days. The expression of surrogate light chain λ5 was detected by FACS. E) Bone marrow cells were isolated from IRF4+/−/−Myc mice and transplanted into the non-irradiated syngenic host mice at 1×10^6 cells per mouse. A total of 10 host mice were used. The host mice were analyzed by FACS after 5 to 10 weeks. Two representative bone marrow analysis were shown. (TIF)

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Author Contributions

Conceived and designed the experiments: SP SM RL. Performed the experiments: SP SM LT. Analyzed the data: SP SM JE SJ RL. Contributed reagents/materials/analysis tools: KW. Wrote the paper: RL.

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