UNIVERSITY OF CALIFORNIA, SAN DIEGO

# The Interferon Pathway Attenuates Acute Myeloid Leukemia Development

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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Committee in charge

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2012

The thesis of Benjamin L Lewin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012

I dedicate this thesis to my friends and family for their love and support.

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I would like to thank Dr. Zhang for the opportunity to join her lab and pairing me with Russ DeKelver. Russ has been a great mentor and friend during my entire time in the Zhang lab. I would also like to thank the entire Zhang lab for their selfless help with my project.

ABSTRACT OF THE THESIS

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The fusion protein AML1-ETO is generated from the translocation t(8;21)(q22;q22) and is identified in over 12% of normal acute myeloid leukemia cases. While AML1-ETO does not induce acute myeloid leukemia in the murine retroviral transduction model, a spliced isoform, AML1-ETO9a, is able to induce

acute myeloid leukemia. One difference between AML1-ETO and AML1-ETO9a is the ability of AML1-ETO to induce an interferon response to a greater degree than AML1-ETO9a. Here I verify that interferons and interferon stimulated genes are transcribed to a greater degree in the presence of AE than AE9a in the U937T cell lines. I also show that AE expression and universal interferon treatment inhibit proliferation in the U937T cell line. Next I verify in mouse bone marrow cells that AE induces interferon stimulated genes more effectively than AE9a. In addition, the induction of interferon stimulated genes is greater in bone marrow cells of C57BL/6 wild type mice than interferon receptor knockout mice. I also show that both global interferon treatment and loss of interferon receptor lead to decreases in proliferation and self-renewal ability. Finally I show that lethally irradiated mice treated with AE9a transduced fetal liver cells from mice without interferon receptor experience enhanced acute myeloid leukemia development compared to mice receiving AE9a transduced fetal liver cells from wild type mice. I also show that similar spleen and liver mass, and leukocyte composition is observed in leukemic mice regardless of the genotype of the donor fetal liver cells.

# I

# Introduction

**Acute myeloid leukemia abnormalities**

Acute myeloid leukemia (AML) is a disease characterized by leukocyte abnormalities such as excessive proliferation and poor differentiation oftentimes due to chromosomal anomalies.[1] In fact, chromosomal translocation abnormalities can be identified in more than half of all AML cases but difficulty in identifying these abnormalities has contributed to the poor 25% five year survival rate.[1,2] A common chromosomal translocation in AML is the 8q22;21q22 (t8;21).[3] The t8;21 translocation is associated with up to 12% of normal AML cases and 40% of the subtype M2 AML cases of the French-American-British (FAB) classification, a subtype associated with maturation of the granulocytic family of white blood cells.[4]

**Characteristics of the t(8;21) related AML**

The t(8;21) translocation involves the acute myeloid leukemia 1 (*AML1*) gene on chromosome 21 and the eight-twenty one (*ETO*) gene on chromosome 8 to generate the fusion gene *AML1-ETO* (*AE*).[4] The *AE* gene contains the 5' portion of the *AML1* gene along most of the coding region from *ETO* and encodes for a 752 amino acid protein.[5] The AML1 portion of the AE fusion protein consists of the DNA-binding runt homology domain (RHD) while the ETO portion contains four Nervy homologous regions (NHR1-4). AML1 is also known as core binding factor-α (CBFα) and forms a heterodimer with core binding factor-β (CBFβ) to transcriptionally activate AML1 target genes, such as the T-cell receptors (TCRs), granulocyte-macrophage colony stimulating factor (GM-

CSF) and $p14^{ARF}$.[6,7] All of these genes have AML1 binding sites in their promoter or in a distal enhancer. Other binding partners of AML1 include factors such as transcription factor PU.1 (PU.1) and core binding factor protein (C/EBPα). The ETO portion of the fusion protein does not bind DNA but it has been thought to be a global gene repressor through interactions with histone deacetylases (HDACs), histone methyltransferases and DNA methyltransferases. Through its NHR domains, ETO recruits certain co-repressors, such as nuclear receptor corepressor (NCoR) and thyroid hormone receptor (SMRT), which in turn recruit HDACs to decrease overall gene expression.[6] In addition to utilizing HDACs, AE has also been thought to silence genes by recruiting DNA methyltransferases (DNMT) since DNMT inhibitors, such as 5-aza-2'-deoxycytidine, helps restore AML1 target gene transcription.[8] DNA histone transferases such as protein arginine methyltransferase 1 (PRMT1) have also been identified in cells expressing leukemic isoforms of AE.[9]

**An alternatively spliced isoform of AE**

An alternatively spliced isoform of the *AE* transcript, *AML1-ETO* (*AE9a)*, encodes for a 575 amino acid protein.[5] *AE9a* differs from *AE* because its protein product is truncated at the C-terminus and contains an extra ETO exon named 9a. The exon 9a encodes for an early stop causing translational termination after exon 8, leaving exons 9, 10 and 11 untranslated. This results in the loss of the two domains, NHR3 and NHR4, from the ETO portion of AE in AE9a.

**Functions of AML1-ETO and AML1-ETO9a**

The AE fusion protein represses expression of AML1 target genes since the RHD domain of AML1 allows for DNA binding of AML1 target genes and the ETO portion of the fusion gene represses expression of the AML1 target genes by recruiting HDACs and other repressors. Some of the notable genes repressed by AE are TCR, GM-CSF and $p14^{ARF}$.[7] GM-CSF is an important factor for myeloid cell development and has recently been shown to be important in AE induced leukemia.[10] The factor $p14^{ARF}$ is a tumor suppressor and has been shown to be directly inhibited by AE at the transcription level.[7]

In addition to decreasing expression of AML1 target genes, AE is able to decrease expression of transcription factors via binding of the RHD domain. Factors such as CBEPα and PU.1 have been identified as transcriptional regulators with decreased activity due to AE interactions.[11,12] AE inhibits the antiproliferative transcription factor CEBPα at its promoter, leading to a block in neutrophil differentiation suggesting a role in leukemogenesis.[13] AE also inhibits the transactivation activity of the transcription factor PU.1 essential for myeloid differentiation in the murine model.[12] Taken together these data suggest AE has serious effects upon genes associated with hematopoiesis and tumor development, ultimately contributing to leukemogenesis.

AE expression also leads to an overall decrease in cell number by inhibiting cell division and promoting apoptosis.[14,15] A decrease in cell number is inconsistent with the excessive leukocyte proliferation observed in AML development. However, blocked differentiation of myeloid, lymphoid and

erythroid lines in established and primary cell lines expressing the AE protein, suggests a role in leukemogenesis.[14,15] The *AE* gene affects gene expression and cell proliferation, but the AE fusion protein alone is not enough to lead to leukemia development.[5] Though AE is unable to induce AML in the murine retroviral transduction-transplantation model, AE9a is able to induce leukemia in this murine model

**The interferon pathway**

Interferons (IFNs) are a class of cytokines first described by Isaac and Lindenmann in 1957 and are important in the function of the human immune system.[16] There are three types of IFNs; IFN type I, type III and type III. The type I IFNs consist of IFN-α and IFN-β.[17] The type I IFNs signal through the receptor Ifnar11/2 to yield their antiviral, antiproliferative and apoptotic effects.[18] This signal is then passed on through a Janus-kinase STAT pathway to have an effect upon gene expression in the nucleus. The type II IFNs are made up of only IFN-γ and this type of IFN exhibits antiviral activity by acting as an immunomodulatory cytokine and signals through the receptor IFNGR1/2. The third group of interferons consists of interleukins (signaling molecules which promote differentiation of both lymphoid and myeloid cells) such as IL-29, IL-28A and IL-28B.[19] The type III interferons also function to protect cells against virus replication through the IL-10 receptor and the Il-28 receptor.

The type I interferons were previously used as a drug to treat AML because of their antiproliferative and immunomodulatory activities. IFN-α has

been shown to be an antineoplastic agent while treating several solid and hematological malignancies.[20] IFN-α has also been shown to be active in treatment of chronic myeloid leukemia (CML), a condition closely related to AML.[21] IFN-α has been used in the clinical setting as a treatment to AML with varying degrees of success. Studies have analyzed methods of IFN-α treatment to AML patients and suggest that continuous delivery of IFN-α is critical to successful treatment.[22] IFN- α treatment of AML remains an attractive alternative due to the poor effectiveness and relatively toxic properties of current AML treatments.

**Role of AE, AE9a and IFN in leukemogenesis**

While published and unpublished microarray data from the Zhang lab at University of California San Diego and from the Speck lab at University of Pennsylvania suggest that in established human blood cell lines, AE causes greater expression of ISGs than AE9a.[23,24] It has already been shown that AE9a can induce AML in the mouse model while AE is unable to do so, but the mechanism of AE9a induced AML remains a mystery. It is possible that AE induces an IFN response which attenuates murine AML while AE9a fails to induce a strong enough IFN response to combat murine AML. It is important to test if expression of IFNs and ISGs can play a role in preventing the leukemic phenotype in primary cell lines, established cell lines and in mice.

While microarray data has shown increased levels of IFN and IFN inducible genes, these results have to be first confirmed by RT-qPCR. We will

use the human blood cell line U937T along with U937T-AE and U937-AE9a with tetracycline inducible promoters. Upon induction of AE and AE9a, we will compare expression of IFN-α, IFN-β and ISGs such as (ISG15 ubiquitin-like modifier) ISG15, (Interferon regulatory factor 7) IRF7, (Interferon induced protein 44) IFI44 and (2'-5'-oligoadenylate synthetase-like protein) OASL by qRT-PCR. We will then perform a proliferation assay on the U937T cell lines treated with IFNs to see if IFN changes the proliferative potential of the cells. In addition, we will test levels of IFN and ISGs in AE or AE9a infected bone marrow cells of both wild type C57BL/6 mice and in IFN-alpha/beta receptor knockout (Ifnar1 KO) C57BL/6 mice. We will perform a colony assay on bone marrow cells and treat with IFN to study the role of IFN upon bone marrow cell proliferation and self-renewal. We will irradiate both wild type C57BL/6 mice and Ifnar1 KO C57BL/6 mice in order to destabilize the immune system and bone marrow reserves. The irradiated mice will then be rescued with bone marrow transplant (BMT) via tail vein injections from either wild type C57BL/6 mice or from C57BL/6 mice infected with AE or AE9a. This will show the importance of both Ifnar1 in preventing AML development. Collectively, these results may explain why AE9a successfully induces murine leukemia and why AE fails.

# II

# Results

**AE expression strongly stimulates the IFN response in U937T cells but AE9a expression still induces the IFN response**

The U937T cell line was generated by fusion of the VP16 transcriptional activating domain to a tetracycline repressor to form a transactivator. [14] When the cell line is cultured in the presence of tetracycline, the transactivator is unable to bind to an inducible promoter responsible for expression of the genes under its control (*AE* and *AE9a*). Upon removal of tetracycline, the transactivator induces expression of *AE* and *AE9a* in the U937T-AE and U937T-AE9a cell line, respectively.

U937T, U937T-AE and U937T-AE9a were cultured in RPMI with 10% FBS. Tetracycline was removed from the media by centrifugation for three minutes at 500*g* followed by aspiration of the supernatant and resuspension of the U937T cells in phosphate buffered saline (PBS). This was performed a total of three times followed by culture of the U937T, U937T-AE and U937T-AE9a cells in tetracycline free media for 48 h. Western blot reveals expression of AE in U937T-AE cells 48h after withdrawal of tetracycline, but lack of expression in U937T-AE cells when cultured in media with 1 µg/mL tetracycline (Figure 1A). Similarly, expression of AE9a is detected in the U937T-AE9a cells 48h after withdrawal of tetracycline but much lower levels of detectable AE9a expression when U937T-AE9a cells were cultured with 1 µg/mL tetracycline. The control U937T cells showed no expression of AE or AE9a under tetracycline or tetracycline free media conditions. The expression of AE and AE9a in U937T-AE

and U937T-AE9a cells cultured in tetracycline free media was confirmed at 48 hrs prior to qPCR analysis of interferon pathway activation.

Analysis of unpublished data from the Zhang lab and the Speck lab suggested *IRF7*, *OASL*, *ISG15* and *IFI44* were some ISGs that are stimulated by AE and AE9a with higher levels of expression in the presence of AE than in AE9a. Comparing levels of gene expression by RT-qPCR revealed that *IRF7*, *OASL*, *ISG15* and *IFI44* expression was indeed increased at the mRNA level after induction of AE or AE9a (Figure 1B). Interestingly, expression of AE led to higher levels of these genes; *IRF7* mRNA levels were increased 49.9 fold after expression of AE but only 8.4 fold after expression of AE9a. Similarly *OASL*, *ISG15* and *IFI44* mRNA levels increased 169.1, 32.8 and 40.5 fold after AE expression but only 31.4 fold, 7.6 fold and 10.3 fold after AE9a expression, respectively. The U937T control cell line showed little change of ISGs expression and revealed that the expression AE and AE9a were responsible for these changes. These results show that both AE and AE9a can induce the IFN evidenced by the increased expression of the four ISGs of interest. Also, AE is more adept at inducing the IFN response than AE9a. Next the levels of type I IFNs in the U937T cell line were measured.

Primers specific for *IFN α* mRNA (set 1 detected IFN α 1, 13 while set 2 detected *IFN α* (1, 6, 10, 13, 16, 17, 21) and for *IFN β* mRNA were used in qRT-PCR to observe levels of transcription induced by AE and AE9a (Figure 1C). Expression of AE led to greater induction of type I IFN than AE9a. Levels of *IFN α* mRNA were 14.5 and 14.9 fold higher after AE expression and were only 7.8

and 7.0 fold higher after AE9a expression. Similarly, levels of *IFN β* mRNA were 16.0 fold higher after AE expression and 8.8 fold higher after AE9a expression. The control cell line U937T had increased type I IFN levels of just under two fold after tetracycline withdrawal, but these levels were much lower than the levels induced by AE and AE9a expression. This data of type I IFN levels along with levels of ISGs confirmed that both AE and AE9a expression evoke the IFN response, at least partially by stimulating type I IFN production. Also, expression of AE leads to a stronger IFN response than AE9a.

**AE expression and IFN treatment leads to inhibition of proliferation in U937T cells**

An MTS assay was performed upon the U937T cell lines after confirmation of induction of ISGs after AE and AE9a expression and treatment of  IFN. This assay utilized the MTS reagent, which is reduced by actively metabolizing cells, and is used to approximate proliferation by spectrophotometer. When U937T was cultured with tetracycline, the absorbance readings at 490 nm were measured and were 1 and 1.81 on day 2 and day 3 after being seeded out at 250,000 cells/mL (Fig. 2A). When the U937T cells were cultured without tetracycline, the readings were 1 and 2.12, showing no decrease of proliferation after tetracycline withdrawal.

There was a decrease in cell proliferation after induction of AE but not AE9a. Absorbance readings of the U937T-AE cultured with tetracycline at day 2 were 0.67 and 1.54, respectively. When U937T-AE cells were cultured without

tetracycline, the absorbance readings were 0.66 and 1.1, showing a decrease in cell proliferation by day 3. The readings of absorbance at 490 nm for the U937T-AE9a cells cultured with tetracycline were 0.97 and 1.44. When the U937T-AE9a cells were cultured without tetracycline the readings were 0.93 and 1.81, showing no change or an increase in cell proliferation after tetracycline withdrawal.

There was a loss of proliferation in the U937T cell lines after treatment with interferon but this loss of proliferation was the same at 500 units and 1000 units of IFN/mL. In addition the loss of proliferation was most easily seen when AE and AE9a cells were cultured without tetracycline. When U937T-AE cells were cultured with tetracycline and 500 or 1000 units/mL interferon, the absorbance readings were 0.63 and 0.64 during day 2 and 1.57 and 1.71 during day 3. When the U937T-AE cells were cultured without tetracycline and 500 or 1000 units/mL interferon, the absorbance readings fell to 0.49 and 0.46 during day 2 and 0.61 and 0.63 during day 3. The decrease in proliferation can be seen by day 2 but is significant by day 3. When U937T-AE9a cells were cultured with tetracycline and 500 or 1000 units/mL universal IFN, the absorbance readings were 0.7 and 0.79 during day 2 and 1.45 and 1.49 during day 3. When U937T-AE9a cells were cultured without tetracycline and 500 or 1000 units/mL universal IFN, the absorbance readings were 0.56 and 0.62 during day 2 and 1.3 and 1.26 during day 3. The differences in proliferation are not as striking as those in the U937T-AE cell line but are still significant.

**AE induces a strong IFN response while AE9a induces a weaker IFN response in C57BL/6 mouse bone marrow cells and both responses are reduced in Ifnar1 KO cells**

Bone marrow cells from C57BL/6 WT and C57BL/6 Ifnar1 KO cells were infected twice with virus made from 293T cells. The virus was produced by transfecting 293T with Ecopac for ecotropic retrovirus production and MIP, MIP-AE or MIP-AE9a. After transfection of 293T cells with Ecopac and MIP-AE, a strong band of AE was detected by western blot with α-HA (Fig. 3A). Also, after transfection of 293T cells with Ecopac and MIP-AE9a, a strong band of AE9a was detected. No such bands were detected when 293T cells were only transfected with Ecopac and MIP.

The infected C57BL/6 WT and C57BL/6 Ifnar1 KO cells were harvested a day after infection. Western blot revealed expression of AE in both C57BL/6 WT and Ifnar1 KO bone marrow cells infected with the supernatant of 293T MIP-AE transfected cells (Fig. 3B). AE9a expression was also detected in both C57BL/6 WT and Ifnar1 KO bone marrow cells infected with the supernatant of 293T MIP-AE9a transfected cells. No bands of AE or AE9a were detected in bone marrow cells infected with the supernatant of 293T MIP transfected cells.

RT-qPCR of the same infected C57BL/6 WT and Ifnar1 KO bone marrow cells reveal that ISGs expression is increased strongly by MIP-AE infection and more weakly by MIP-AE9a. The expression of ISGs is lower in Ifnar1 KO cells compared to C57BL/6 cells but is not lost completely. In C57BL/6 WT bone marrow MIP-AE infected cells (C57BL/6 WT-AE), Lymphocyte antigen 6a (*Ly6a*)

mRNA levels increased 6.3 fold, *Oasl2* mRNA levels increased 9.2 fold, *Oasl1* mRNA levels increased 6.8 fold, *Irf7* mRNA levels increased 5.8 fold and IFN γ induced GTPase (*Igtp*) mRNA levels increased 1.5 fold (Fig. 3C). These levels of transcription only increased by 2.3 fold for *Ly6a*, 5.3 fold for *Oasl2*, 3.4 fold for *Oasl1*, 3.2 fold for *Irf7* and 1.4 fold for *Igtp* when the C57BL/6 WT bone marrow cells were infected with MIP-AE9a instead (C57BL/6 WT-AE9a). When C57BL/6 Ifnar1 KO bone marrow cells were infected with MIP-AE (C57BL/6 Ifnar1 KO-AE), the levels of transcription only increased 2, 2.2, 2.4, 2 and 0.4 fold, for the same genes as above. Similarly, after C57BL/6 Ifnar1 KO bone marrow cells were infected with MIP-AE9a (C57BL/6 Ifnar1 KO-AE9a), levels of transcription increased 1.3, 1.7, 1.3, 1.2 and 0.6 fold. These results show that AE induces stronger expression of ISGs than AE9a in mouse bone marrow cells and that this induction is partially lost in Ifnar1 KO bone mouse bone marrow cells

**Proliferation and self-renewal of bone marrow cells is inhibited weakly by addition of universal IFN and by loss of Ifnar1**

C57BL/6 WT-AE, C57BL/6 WT-AE9a, C57BL/6 Ifnar1 KO-AE and C57BL/6 Ifnar1 KO-AE9a cells were plated onto M3434 methylcellulose for a cell colony assay. The C57BL/6 WT and Ifnar1 KO bone marrow cells infected with MIP were also plated onto M3434 methylcellulose for a cell colony assay but

were unable to form colonies more than one week after selection and numbered 15,000 cells (Fig 4A). C57BL/6 WT-AE cells formed ten colonies (280 thousand cells) the first week after selection, but by week three there were 173 colonies (4 million cells). Treatment of these C57BL/6 WT-AE cells with universal IFN led to eight colonies formed (917 thousand cells) the first week after selection, and the formation of 88 colonies (2.85 million cells) three weeks after selection. Interestingly, the C57BL/6 Ifnar1 KO- AE formed four colonies (205 thousand cells) one week after selection and 23 colonies (500 thousand cells) three weeks after selection.

C57BL/6 WT-AE9a cells formed 91 colonies (3.5 million cells) one week after selection and 203 (3.2 million cells) colonies three weeks after selection .When the C57BL/6 WT-AE9a cells were treated with universal IFN, 55 (3.6 million cells) and 133 (3.1 million cells) colonies were formed one and three weeks after selection, respectively. C57BL/6 Ifnar1 KO-AE9a cells formed 60 colonies (3 million cells) one week after selection and 93 colonies (2 million cells) three weeks after selection. These results taken together indicate that AE and AE9a give bone marrow cells similar self-renewal and proliferation abilities, but that the self-renewal and proliferation is weaker in cells with AE compared to AE9a. Also, universal IFN treatment of bone marrow cells leads to decreased ability of self-renewal. Finally, loss of Ifnar1 also leads to decreased ability to self-renew, especially for cells infected with AE.

Only one trail of the colony formation assay begun early enough to have data up to the fifth replating. Five weeks after selection, C57BL/6 WT-AE cells

formed 155 colonies (3.5 million cells) (Fig. 4B). C57BL/6 WT-AE treated with universal IFN formed 199 colonies (2.9 million cells) five weeks after selection. The C57BL/6 Ifnar1 KO-AE cells formed 134 colonies (2.7 million cells) five weeks after selection. Also, C57BL/6 WT-AE9a cells formed 66 colonies (2 million cells) 5 weeks after selection. Upon treating C57BL/6 WT-AE9a cells with universal IFN, 69 colonies (750 thousand cells) were formed after 5 weeks. C57BL/6 Ifnar1 KO-AE9a cells formed 194 colonies (3.7 million cells). These results suggest that the block of proliferation and self-renewal by AE and loss of Ifnar1 may be only temporary. Also, this suggests that IFN treatment may only work in the short term to block proliferation and self-renewal.

**Mice receiving Ifnar1 KO BMT experience rapid death from leukemia**

To examine the leukemic potential of AE and AE9a, mice were lethally irradiated and BMT was performed with fetal liver cells (FLC) infected with MIG, MIG-AE or MIG-AE9a. The survival of mice 20 weeks after receiving C57BL/6 WT FLC infected with MIG, MIG-AE and MIG-AE9a was 93%, 77% and 84%, respectively (Fig. 5A). The survival of mice 20 weeks after receiving C57BL/6 Ifnar1 KO FLC infected with MIG, MIG-AE and MIG-AE9a was 85%, 80% and 27%, respectively. Mice receiving cells infected with MIG-AE9a were the only mice to develop leukemia as identified by enlarged spleen and liver, FACS analysis and cell morphology. This confirms that AE9a is able to induce leukemia in the murine BMT model and that the Ifnar1 may be an important factor in preventing leukemia.

**Recipients of C57BL/6 WT and Ifnar1 KO FLC develop leukemia with similar profiles**

Mice developing leukemia both displayed hepatosplenomegaly and similar percentages of blood cell markers in peripheral blood. The average mass of a leukemic mouse's spleen and liver receiving C57BL/6 WT MIG-AE9a FLC was 0.36 g and 0.51 g respectively (Fig. 6A). The average mass of a mouse spleen and liver receiving C57BL/6 Ifnar1 KO MIG-AE9a FLC was 1.25 g and 1.628 g respectively. These results indicate that both the spleen and livers were enlarged for leukemic mice and were similar in weight for C57BL/6 WT and Ifnar1 KO mice.

FACS analysis was performed on spleen cells, bone marrow cells and peripheral blood cells to determine the makeup of the white blood cells in leukemic mice. Analysis of GFP+ peripheral blood cells revealed that there were 2.7% CD3+ (T cell marker) cells and 0.6% B220+ cells in mice receiving C57BL/6 WT AE9a infected FLC (Fig. 6B) The percentages of CD3+ and B220+ cells in mice receiving C57BL/6 Ifnar1 KO AE9a infected FLC were 1.9% and 1.5% respectively. The percentages of Gr-1+ or CD11b+ cells in mice receiving C57BL/6 WT AE9a infected FLC was 1.9% and was 3.0% in mice receiving C57BL/6 Ifnar1 KO AE9a infected FLC. In addition, mice receiving C57BL/6 WT AE9a infected FLC had 52.8% c-kit+ Sca-1- and 15.1% c-kit+ and Sca-1+ cells. The mice receiving C57BL/6 Ifnar1 KO AE9a infected FLC had 53.6% c-kit+ Sca-1- and 13.4% c-kit+ Sca-1+ cells.

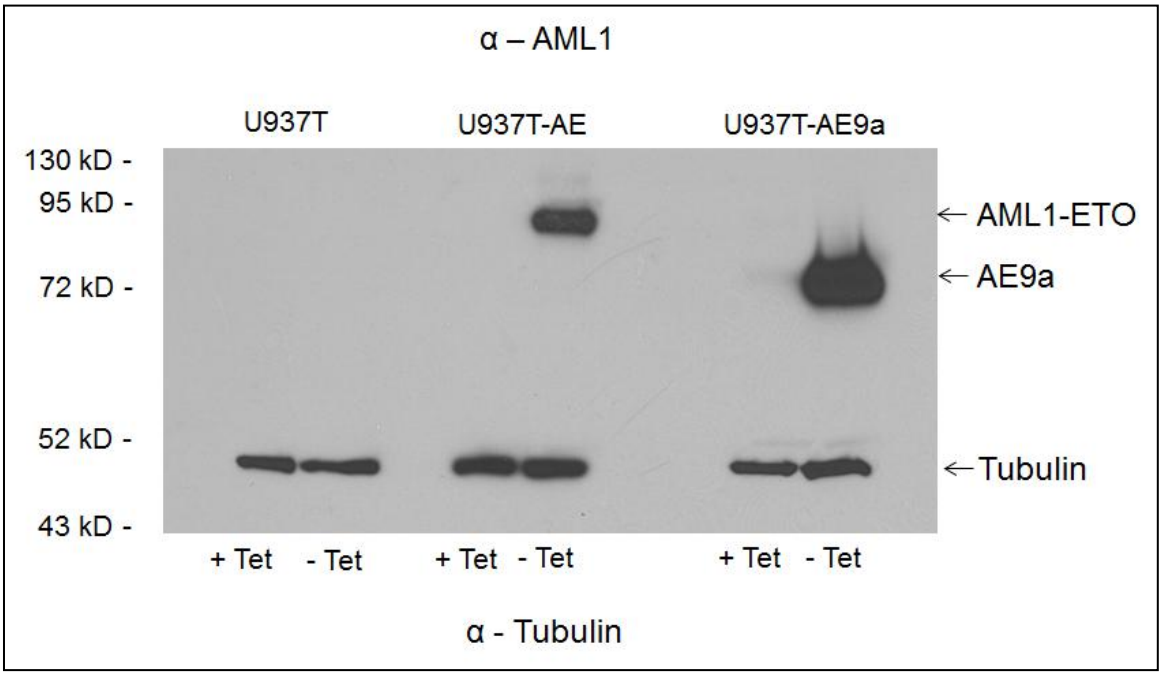
Analysis of GFP+ bone marrow cells confirmed similarities of hematopoietic cells in leukemic mice receiving different AE9a infected FLC. In the mouse receiving FLC from C57BL/6 WT AE9a mice, 50.4% and 13.7% of bone marrow cells were c-kit+ Sca-1- and c-kit+ Sca-1+, respectively. In the mouse receiving FLC from C57BL/6 Ifnar1 KO AE9a mice, 55.3% and 9.0% of the bone marrow cells were c-kit+ Sca-1 and c-kit+ Sca-1+, respectively. These results reveal that the characteristics of leukemia experienced by C57BL6 WT and Ifnar1 KO AE9a recipients were similar.

**Figure 1. AE expression induces a stronger IFN response than AE9a in U937T cell line**

(A) Western Blot of U937T, U937T-AE and U937T-AE9a cells cultured with RPMI and 10% FBS with or without tetracycline for 48 h.

(B-C) RT-qPCR of U937T, U937T-AE and U937T-AE9a cells. The cells were cultured in RPMI and 10% FBS with or without tetracycline for 48 h. RNA was harvested from the cells and 1 ug of RNA was reverse transcribed to cDNA. SYBR green reagent was used for RT-qPCR and the $C_t$ values for the genes of interest were first normalized to GAPDH. The $C_t$ values of the samples without tetracycline were then normalized to those with tetracycline.

A


α – AML1
U937T
U937T-AE
U937T-AE9a
130 kD -
95 kD -
72 kD -
52 kD -
43 kD -
← AML1-ETO
← AE9a
←Tubulin
+ Tet
- Tet
+ Tet
- Tet
+ Tet
- Tet
α - Tubulin

B

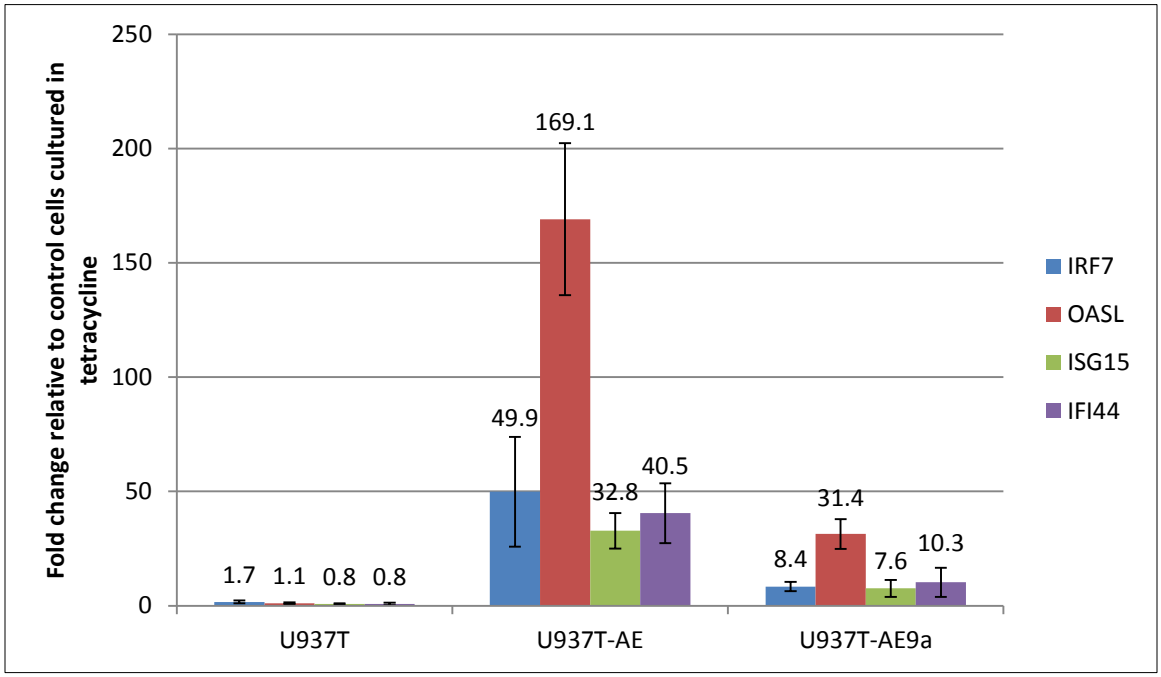



Figure 1 Cont.

C

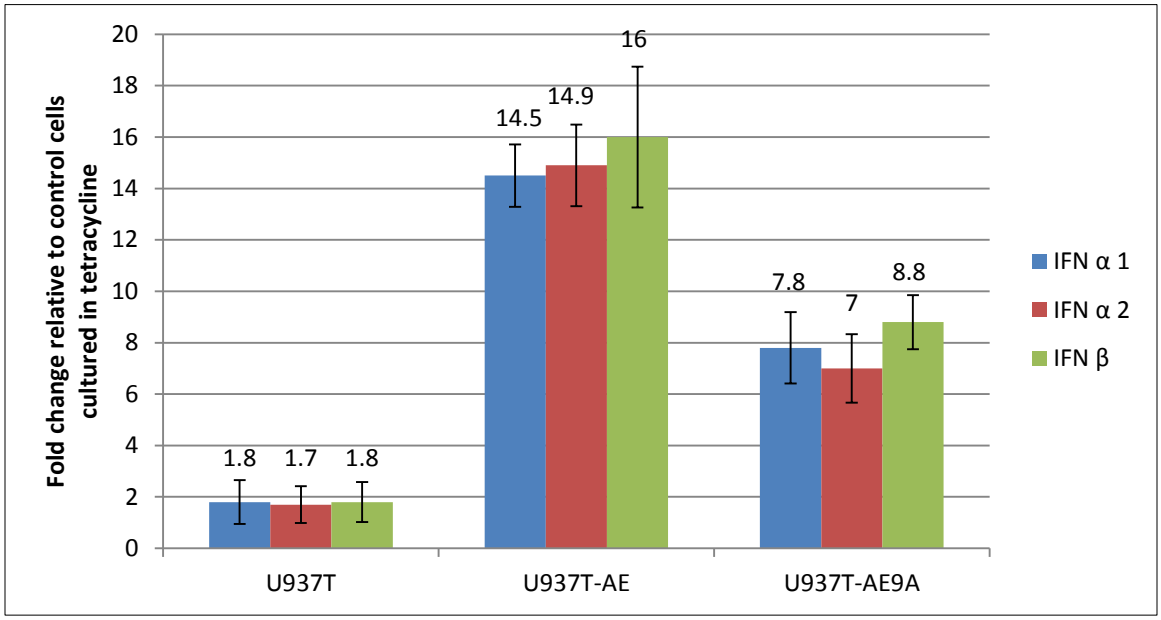



Figure 1 Cont.

**Figure 2. AE and IFN decrease cell proliferation**

(A) U937T cell line all seeded out at an initial concentration of 100,000 cells/mL. Cells were treated with or without tetracycline. Cells were also treated with no IFN, 500 units of IFN or 1000 units of IFN. MTS assay used to measure proliferation.

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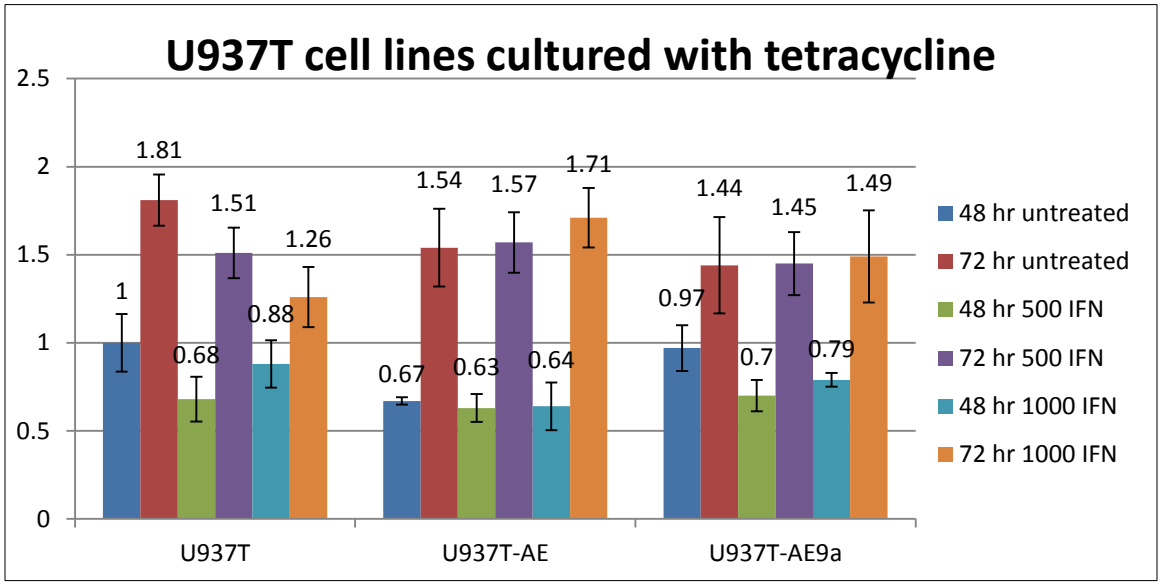
A


U937T cell lines cultured with tetracycline
2.5
2
1.5
1
0.5
0
1.81
1.51
1.26
1
0.68
0.88
1.54
1.57
1.71
0.67
0.63
0.64
1.44
1.45
1.49
0.97
0.7
0.79
U937T
U937T-AE
U937T-AE9a
48 hr untreated
72 hr untreated
48 hr 500 IFN
72 hr 500 IFN
48 hr 1000 IFN
72 hr 1000 IFN

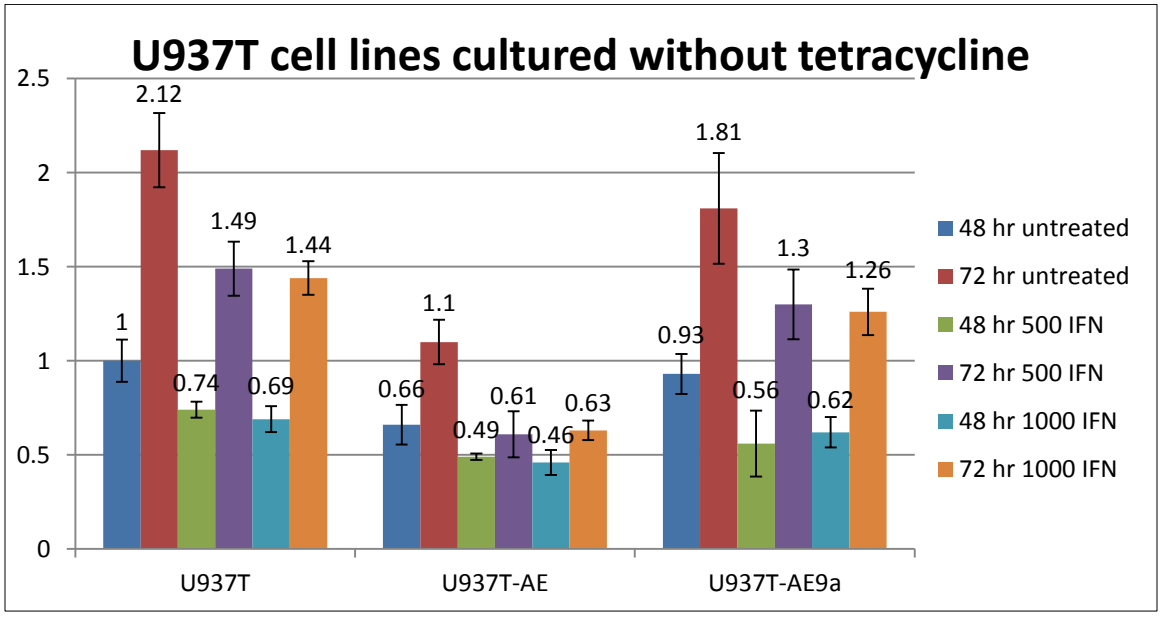

U937T cell lines cultured without tetracycline
2.5
2
1.5
1
0.5
0
2.12
1.49
1.44
1
0.74
0.69
1.1
0.66
0.61
0.49
0.46
0.63
1.81
1.3
1.26
0.93
0.56
0.62
U937T
U937T-AE
U937T-AE9a
48 hr untreated
72 hr untreated
48 hr 500 IFN
72 hr 500 IFN
48 hr 1000 IFN
72 hr 1000 IFN

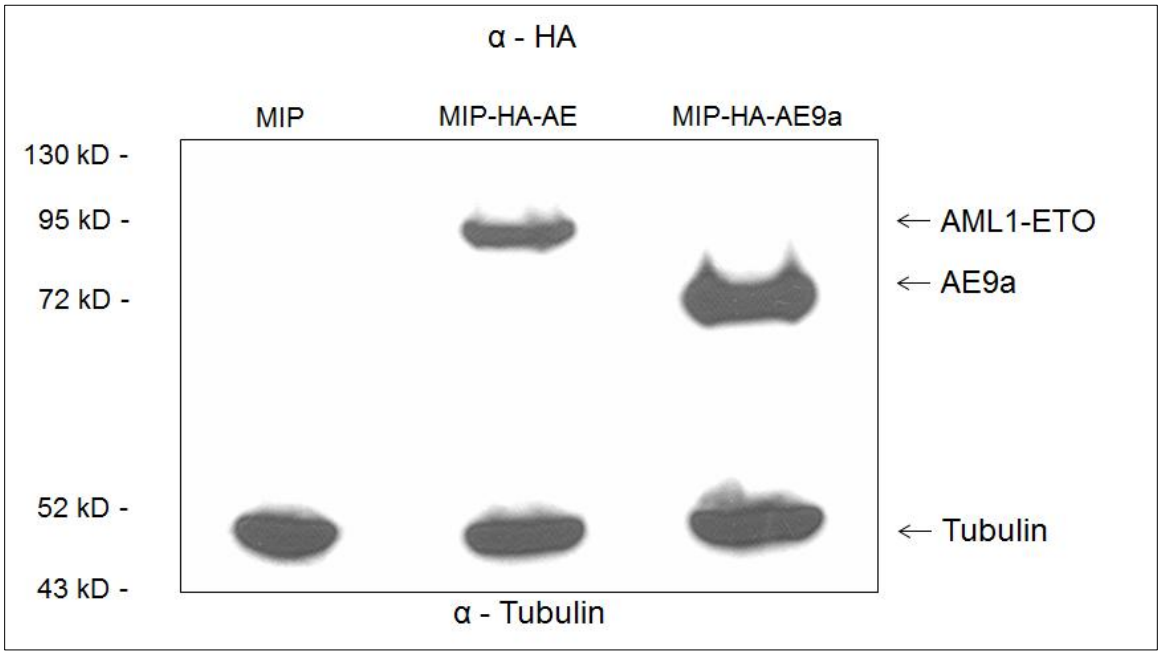
**Figure 3. AE expression induces a stronger IFN response than AE9a in mouse bone marrow cells and the IFN response is partially lost in cells that are Ifnar1 KO**

(A) Transfection of Ecopac with MIP, MIP-HA-AE and MIP-HA-AE9a into 293T cells. Samples harvested 36 hr post transfection.

(B) Infection of C57BL/6 WT and Ifnar1 KO bone marrow cells by retrovirus produced in 293T cells in Fig 3A.

(C) RT-qPCR of C57BL/6 WT and Ifnar1 KO bone marrow cells infected a retrovirus for MIP, MIP-HA-AE and MIP-HA-AE9a. Cells were infected two days in a row and selection in 2 µg/ml puromycin was started the 24 hr after the second infection and the cells were harvested 48 hrs later. RNA was extracted and 0.5 µg of RNA reverse transcribed to cDNA. $C_t$ values normalized to GAPDH and then levels of MIP in C57BL/6 WT and C57BL/6 Ifnar1 KO mice.

A


α - HA
MIP
MIP-HA-AE
MIP-HA-AE9a
130 kD -
95 kD -
72 kD -
52 kD -
43 kD -
← AML1-ETO
← AE9a
← Tubulin
α - Tubulin

B

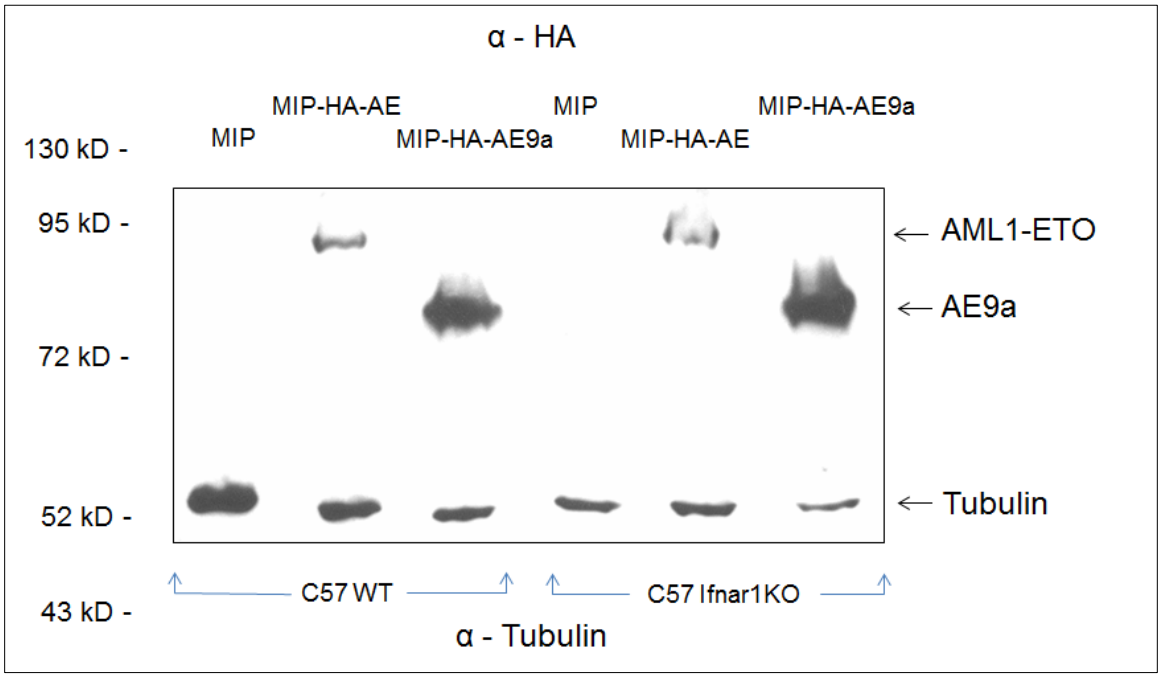



Figure 3 Cont.

C

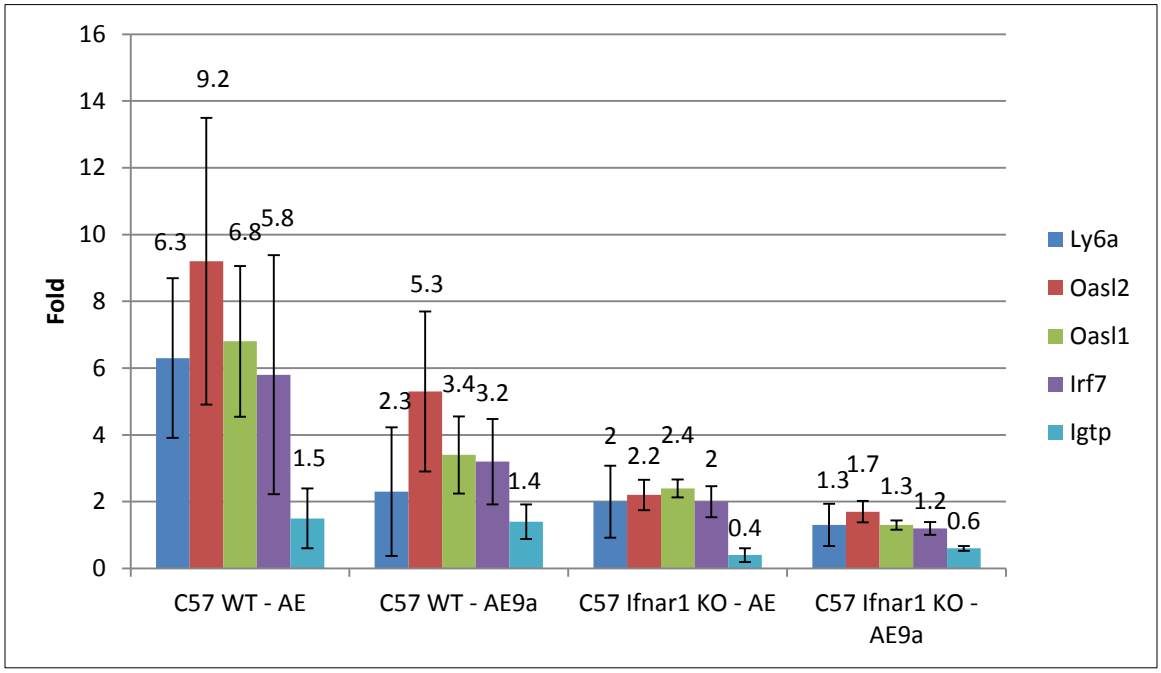


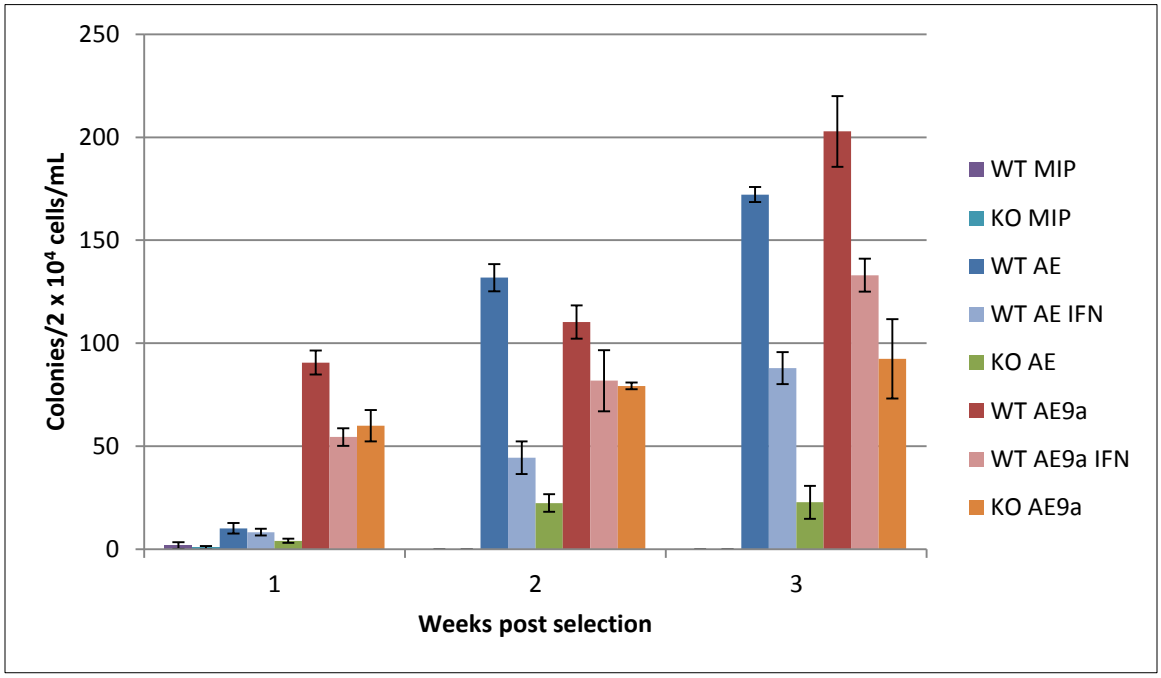

Figure 3 Cont.

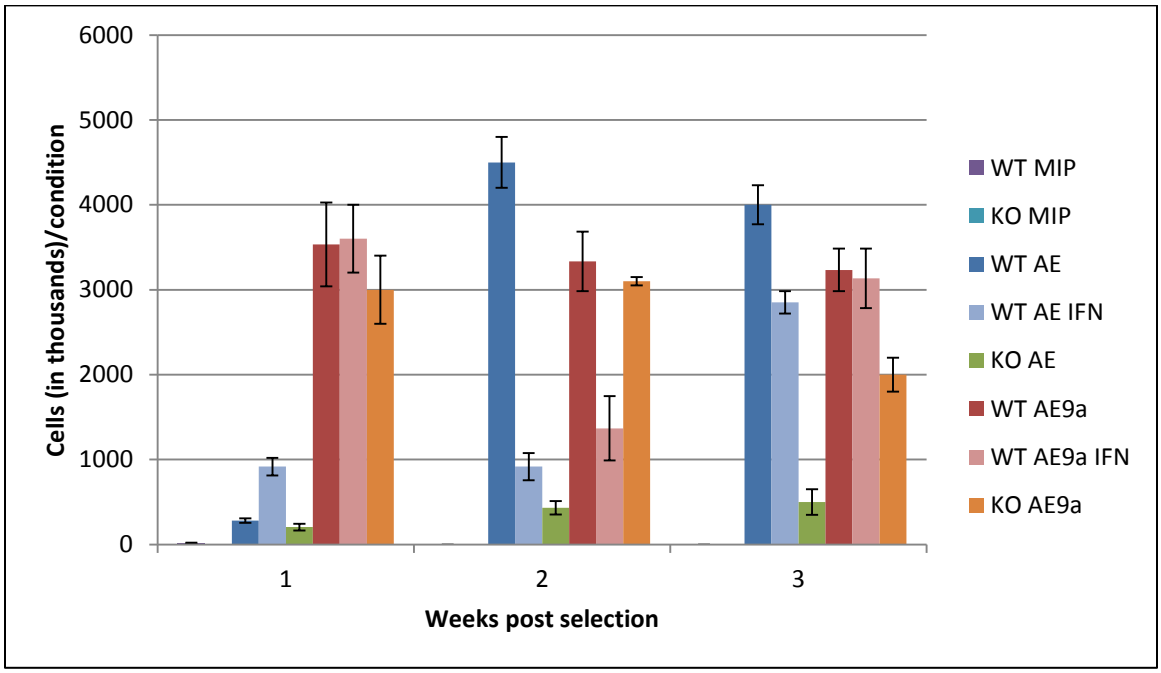
**Fig 4. Proliferation and self-renewal ability of MIP-AE and MIP-AE9a infected bone marrow cells is inhibited by IFN and loss of Ifnar1**

(A) Combined data from three separate colony assays of C57BL/6 WT and Ifnar1 KO bone marrow cells infected with MIP, MIP-AE or MIP-AE9a either untreated or treated with 500 units/mL of IFN.

(B) Data from one colony assay of C57BL/6 WT and Ifnar1 KO bone marrow cells infected with MIP, MIP-AE or MIP-AE9a either untreated or treated with 500 units/mL of IFN.

A


250
200
150
100
50
0
Colonies/2 x 10⁴ cells/mL
1
2
3
Weeks post selection
WT MIP
KO MIP
WT AE
WT AE IFN
KO AE
WT AE9a
WT AE9a IFN
KO AE9a


6000
5000
4000
3000
2000
1000
0
Cells (in thousands)/condition
1
2
3
Weeks post selection
WT MIP
KO MIP
WT AE
WT AE IFN
KO AE
WT AE9a
WT AE9a IFN
KO AE9a

B

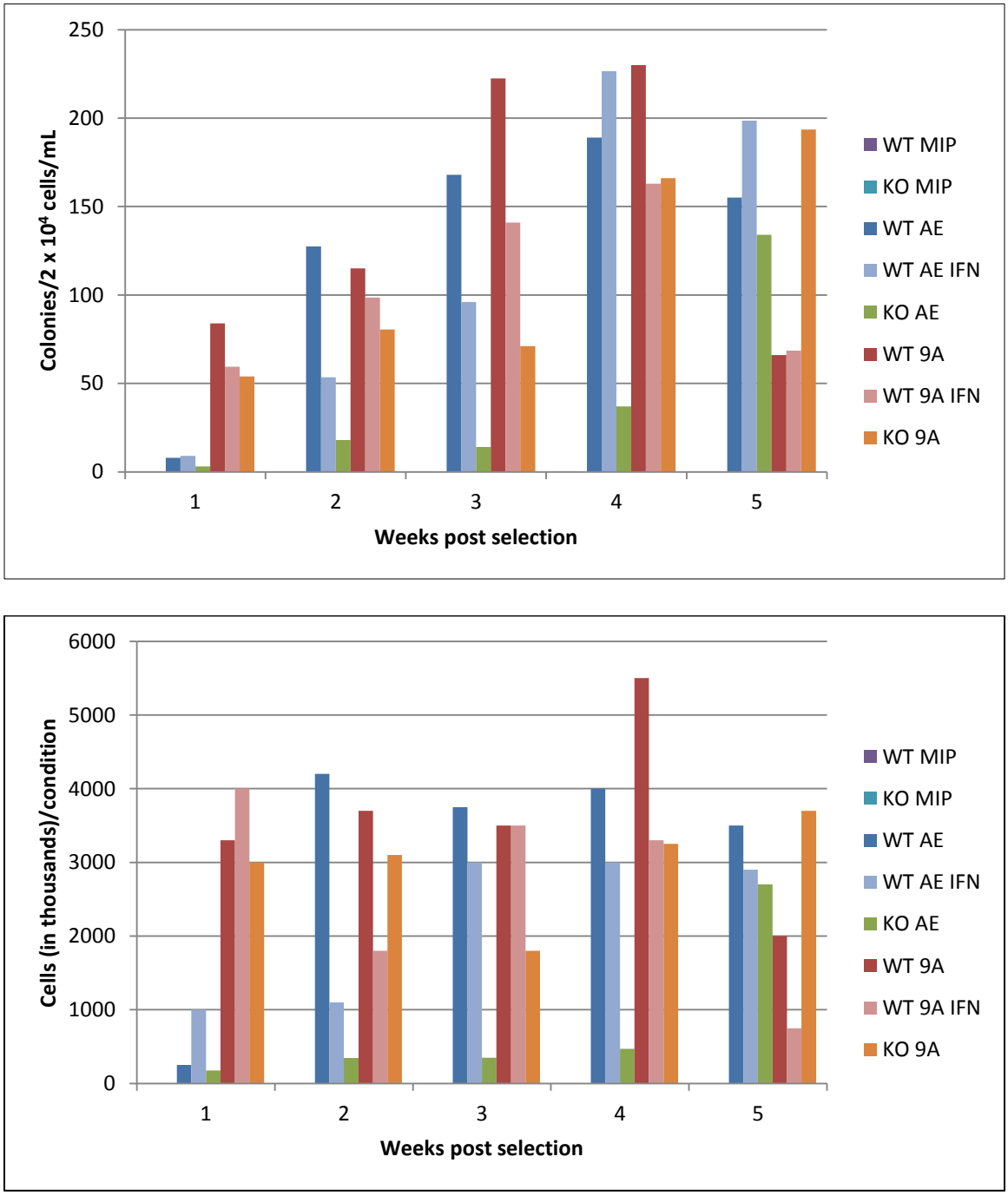



Figure 4 Cont.

**Figure 5. Mouse recipients of Ifnar1 KO AE9a infected fetal liver cells experience rapid leukemogenesis**

(A) Survival curve of irradiated mice rescued with bone marrow transplant from C57BL/6 WT or Ifnar1 KO mouse fetal liver cells infected with MIG, MIG-AE or MIG-AE9a.

A

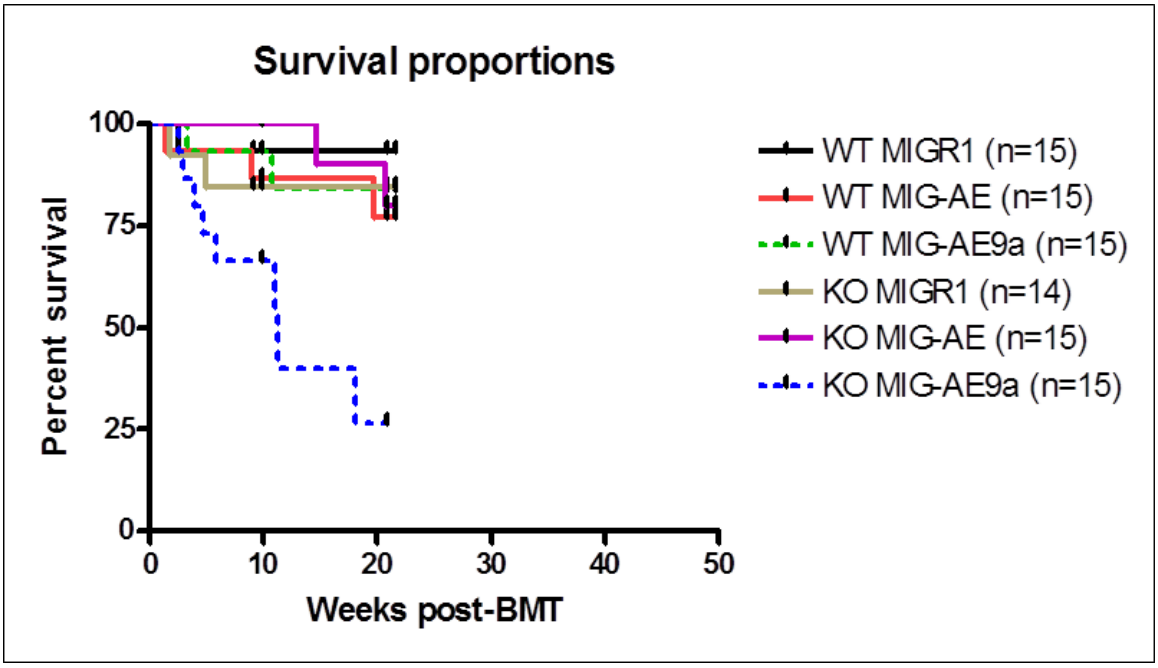
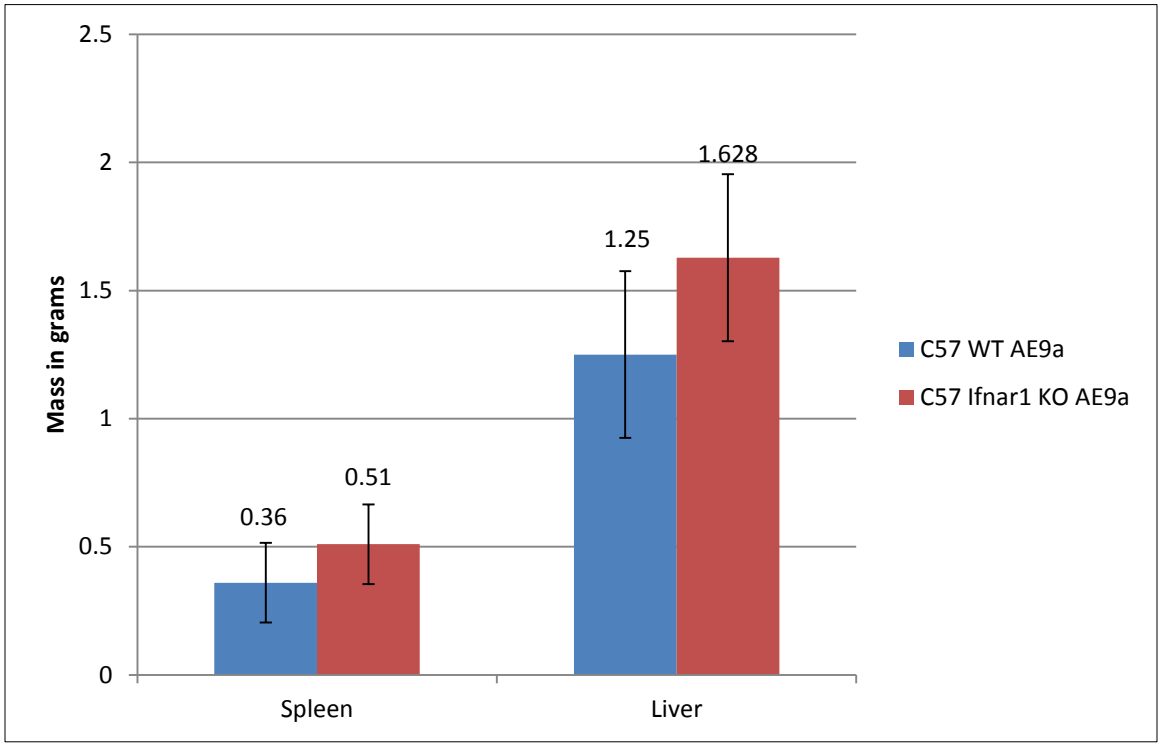

Survival proportions
Percent survival
100
75
50
25
0
0
10
20
30
40
50
Weeks post-BMT
WT MIGR1 (n=15)
WT MIG-AE (n=15)
WT MIG-AE9a (n=15)
KO MIGR1 (n=14)
KO MIG-AE (n=15)
KO MIG-AE9a (n=15)

**Figure 6. Comparison of C57BL/6 WT and Ifnar1 KO leukemic mice**

(A) Average mass of spleen and liver of C57BL/6 WT and Ifnar1 KO leukemic mice. For C57BL/6 Ifnar1 KO and WT mice n=5 and n=2 respectively.
(B) Percentage of GFP+ cells with certain blood cell markers. For C57BL/6 Ifnar1 KO and WT mice n=5 and n=2 respectively.
(C) Single stain controls of FACS analysis of a C57BL/6 Ifnar1 KO leukemic mouse for the markers c-kit and Sca-1.
(D) Example of FACS analysis of GFP+ cells from C57BL/6 WT and Ifnar1 KO leukemic mice with blood cell markers c-kit and Sca-1.

A


2.5
2
1.5
1
0.5
0
Mass in grams
0.36
0.51
1.25
1.628
Spleen
Liver
C57 WT AE9a
C57 Ifnar1 KO AE9a

B

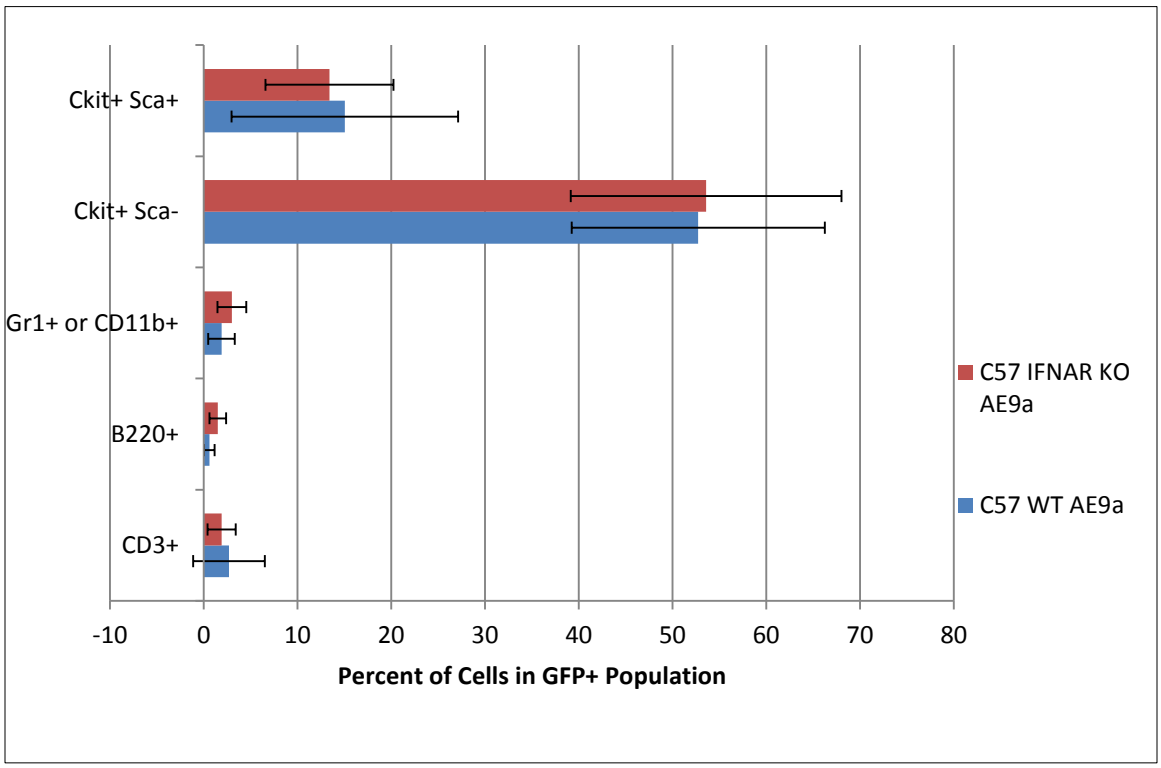



Figure 6 Cont.

C

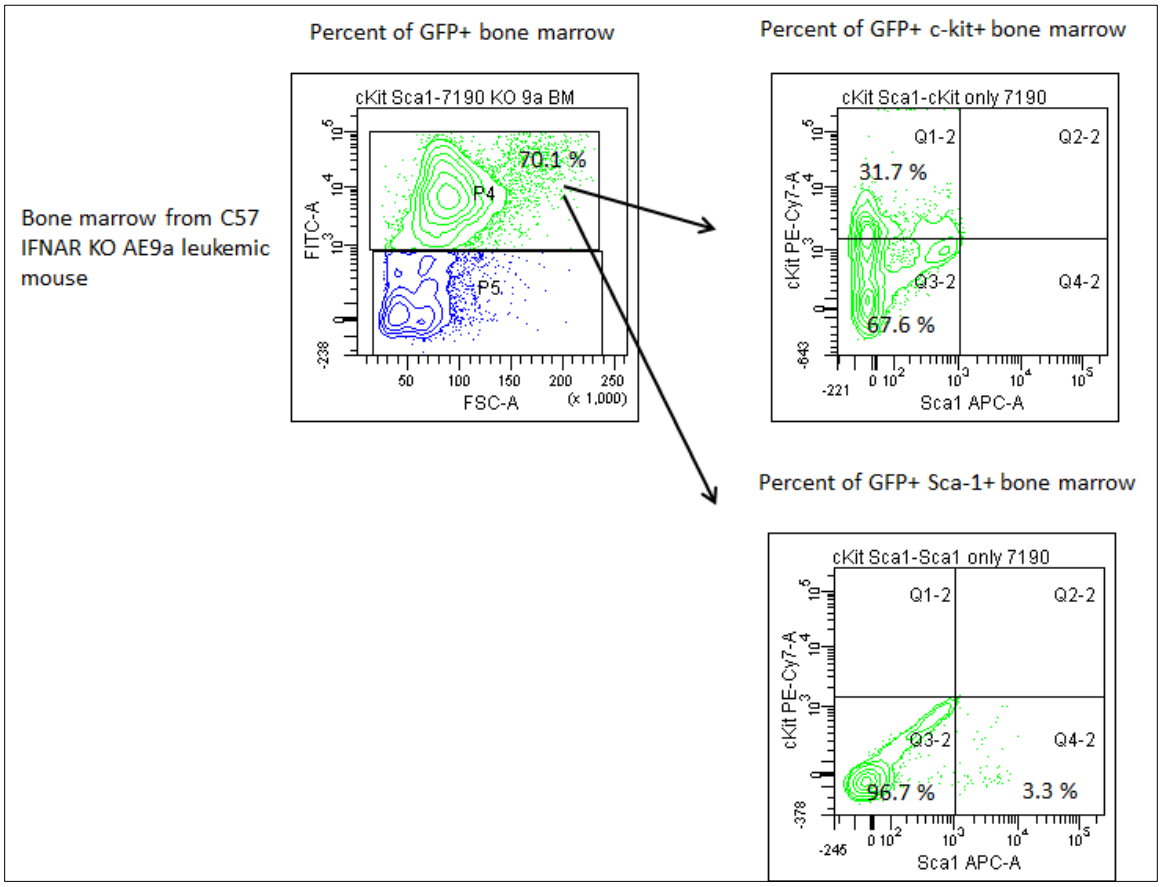



Figure 6 Cont.

D

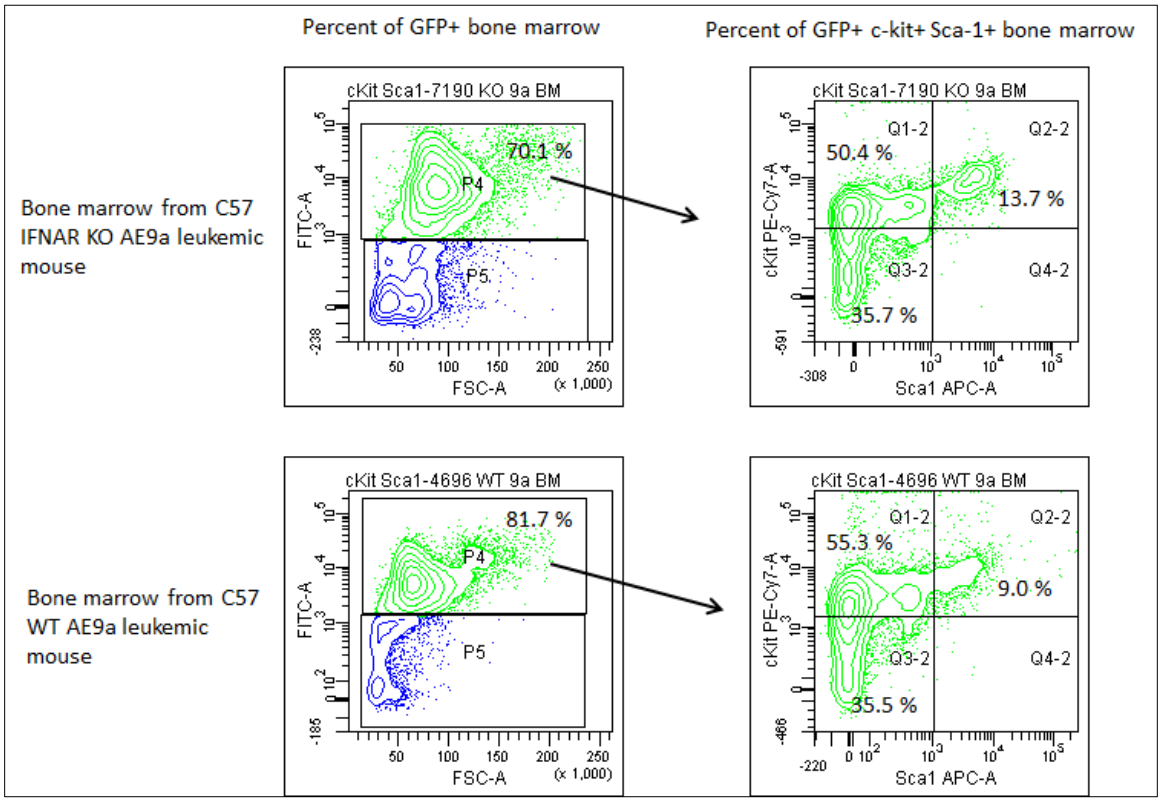



Figure 6 Cont.

# III

# Discussion

I have used the human U937T myeloid cell line to show that stable expression of both AE and AE9a will induce the interferon response (Fig. 1A-C). With an MTS assay, I have shown that AE expression and universal IFN treatment inhibit proliferation of U937T cells (Fig. 2A). I have also shown that the interferon response can be induced in primary bone marrow cells transduced with AE and AE9a (Fig. 3A-C). Through a colony formation assay I revealed the effects of stable AE and AE9a expression and interferon pathway stimulation upon the proliferation and self-renewal of mouse bone marrow cells (Fig. 4A-B). I revealed that while retroviral-transduction of AE9a induces leukemia in mice receiving FLC from C57BL/6 WT mice, the onset of leukemia is accelerated in mice receiving FLC from C57BL/6 Ifnar1 KO mice (Fig. 5A). Finally I show that leukemic mice receiving AE9a FLC cells from C57BL/6 WT or Ifnar1 KO mice develop leukemia with similar characteristics (Fig. 6A-D).

**Interferon response induced after AE and AE9a expression**

In the U937T cell line, the interferon response was activated after expression of AE and AE9a (Fig 1A-C). A stronger response was observed with expression of AE, but AE9a still induced the interferon response. This is consistent with the observations that murine retroviral-transduction of AE9a but not AE lead to the onset of leukemia since IFNs are antiviral, antitumor, antiproliferative and immunomodulatory molecules. The decreased IFN response in cells expressing AE9a could partially explain the increased leukemic potential of AE9a compared to AE.

It remains to be seen why AE and AE9a both stimulate the interferon response. AE is usually a repressor of transcription and the fact that the interferon pathway is an exception to the normal way in which AE acts is fascinating. It has been shown that AE9a is a less repressive factor because of the loss of the NHR3 and NHR4 domains which recruit HDACs in order to repress target genes. This raises the question whether the NHR3 and NHR4 domains work to stimulate the IFN response through HDACs or a different mechanism? If AE works to stimulate the interferon response through HDACs, it might do so by recruiting HDACs to repressors of the interferon pathway.

Similar induction of the IFN response by AE and AE9a was also observed in bone marrow of C57BL/6 mice (Fig. 3A-C). This response was high in bone marrow of C57BL/6 wild type mice but was decreased in bone marrow C57BL/6 Ifnar1 KO mice. This suggests that both AE and AE9a work through an Ifnar1 dependent pathway to activate the IFN response but further studies are needed to confirm this. Since AE and AE9a still activate the IFN pathway in cells from Ifnar1 KO mice, this also suggests that AE and AE9a also work through an Ifnar1 independent pathway to activate the IFN response. The question that remains is which pathway(s) are responsible for IFN pathway stimulation that can account for the IFN response stimulation in Ifnar1 KO bone marrow cells. While IFN expression is typically dependent upon type I IFN binding to Ifnar1 and activation of the JAK-STAT pathway, there are other facets through which ISGs can become highly expressed.

**Ifnar1 KO AE9a mice develop leukemia rapidly**

Lethally irradiated C57BL/6 mice receiving BMT from C57BL/6 Ifnar1 KO FLC transduced with AE9a develop leukemia more rapidly than mice receiving FLC from the WT counterparts (Fig. 5A). This suggests that activation of Ifnar1 by IFN and subsequent activation of the JAK-STAT pathway is an important factor in delaying leukemia in a mammalian organism. Mice receiving BMT from AE9a transduced Ifnar1 KO FLC model as a way to study the effects of leukemia when the IFN pathway is decreased but it is important to show the effects when the IFN pathway is increased. For this, a suitable model would be AE9a transduced FLC from UBP43 KO mice since UBP43 KO mice display increased activation of IFN pathway.

In addition, while mice receiving BMT from Ifnar1 KO FLC transduced with AE9a have developed leukemia, only a few positive control mice receiving BMT from C57BL/6 WT-AE9a have developed leukemia. In another report by Yan et al., the median survival of C57BL/6 mice recipients of FLC transduced with AE9a was 25 weeks.[7] The C57BL/6 WT-AE9a mice will have to continue to be monitored until death by leukemia to validate the rapid onset of leukemia observed by C57BL/6 Ifnar1 KO recipient mice.

**Leukemic mice transduced with C57BL/6 WT or Ifnar1 KO AE9a FLC display similar traits**

Mice that became leukemic due to BMT of AE9a transduced C57BL/6 WT or Ifnar1 KO cells displayed similar leukocyte composition and spleen and liver

weight (Fig. 6A-D). The spleen and liver of the recipients of C57BL/6 Ifnar1 KO-AE9a seemed to be slightly larger than their WT counterparts, but the difference was not statistically significant. The same was true for the percentage of GFP+ peripheral blood cells with certain blood cell markers such as c-kit, Sca-1, Gr-1, CD11b, B220 and CD3. The percentage of markers in GFP+ bone marrow cells was also consistent between the recipients of the two genotypes. This suggests that the same type of leukemia is developed in BMT recipients of C57BL/6 WT-AE9a and Ifnar1 KO-AE9a cells but is simply accelerated in recipients of C57BL/6 Ifnar1 KO-AE9a.

**The IFN pathway inhibits proliferation in the U937T cell line but its role in bone marrow cells is unclear**

In the U937T cell line system, treatment of cells with IFN leads to a block of proliferation (Fig 2A). In fact, after induction of AE9a and treatment with 1000 units/mL of IFN, absorbance levels were 0.62 and 1.26, very similar to the absorbance readings after induction of AE but without treatment of IFN, 0.66 and 1.1. In addition, when AE9a is induced but there is no IFN treatment, measured absorbance levels are higher at 0.93 and 1.81. It seems that IFN treatment does decrease proliferation in cells expressing AE9a to levels near cells expressing AE. This is useful because BMT recipient mice of FLC transduced with AE don't develop leukemia but those recipients of AE9a transduced FLC do develop leukemia. It is possible that this is in part because of the inhibition of proliferation by AE.

In mouse bone marrow cells, treatment with IFN reduced ability for both C57BL/6 WT and Ifnar1 KO bone marrow cells to self-renew and proliferate (Fig. 4A-B). Also, between C57BL/6 WT and Ifnar1 KO bone marrow cells, the loss of Ifnar1 led to reduced ability of bone marrow cells to self-renew and proliferate. The inhibition of self-renewal and proliferation by IFN treatment is expected as IFN are both antitumor and anti-proliferative agents. The greater inhibition by loss of Ifnar1 is surprising as poor activation of the IFN pathway should lead to increased proliferation and increased risk of tumor development. It's possible that Ifnar1 is responsible for more than stimulation of ISGs responsible for these effects in C57BL/6 mice and that another pathway is being affected by the loss of Ifnar1.

# IV

# Materials and Methods

**Cell Culture**

U937T, U937T-AE and U937T-HA-AE9a cells were cultured in RPMI medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine and 1 ug/mL tetracycline. The cells were cultured in a 37° C incubator with 5% carbon dioxide. The cell lines were established as previously described.[14]

293T cells were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine.

Primary mouse bone marrow and fetal liver cells were cultured in Isocove's Modified Dulbecco's Medium (IMDM) with 4% IL3-CM and 4% SCF-CM (2x BM) prior to infection.

**Western Blot**

Cell samples were washed in PBS and resuspended in sample buffer containing 160 mM Tris pH 6.8, 6.4% sodium dodecyl sulfate (SDS), 32% glycerol, 0.032% bromophenol blue and 20% b-mercaptoethanol. After SDS-polyacrylamide gel electrophoresis (PAGE), the proteins were transferred to a nitrocellulose membrane. The membrane was blocked in 5% dry milk in phosphate buffered saline with 0.1% Tween 20 (PBS-T). The blot was then washed with PBS-T and incubated with primary antibodies overnight. Antibodies used included α-AML1, α-tubulin and α-HA-HRP. Following primary antibody incubation, the blot was washed with PBS-T and incubated with the

corresponding secondary antibodies. After treatment with luminol and oxidizing substrates, the blot was visualized on Blue Devil film.

**Retrovirus and Infection**

To produce retrovirus, 293T cells were transfected with 5 µg of MSCV-IRES-PURO (MIP), MIP-AE, MIP-AE9a, MSCV-IRES-GFP (MIG), MIG-AE or MIG-AE9a vectors and with 5 ug of Ecopac vector by PEI reagent with Optimem. The 293T media was changed from DMEM to IMDM again 8 hrs post transfection. Retrovirus supernatants were harversted 48 hours after transfection and were filtered through a 0.45 um filter. The retrovirus supernatant from MIP and MIG vectors was added to recipient bone marrow cells and fetal liver cells, respectively, along with 4% IL3-CM, 4% SCF-CM, 1% HEPES and 1 µL/mL polybrene. The infected cells were centrifuged at 3000 x *g* for 3 hr at 30° C then incubated at 37° C with 5% carbon dioxide. Infections were performed twice on consecutive days for all cells. The cells infected with MIG constructs were identified by their fluorescence after FACS analysis. The cells infected with MIP were identified via puromycin selection.

**RT-qPCR**

RNA was harvested from cells with Qiagen's RNeasy Mini Kit. RNA was reverse transcribed into cDNA using Invitrogen's Super Script III Reverse Transcriptase. 1 µL of cDNA from each sample was used with KAPA SYBR 2x

mix on Biorad's CFX Connect™ Real-Time PCR Detection System. All $C_t$ results were normalized to GAPDH. The forward and reverse primers used to detect genes via RT-qPCR were: hGAPDH (5'-GAA GGT GAA GGT CGG AGT C-3' 5'-GAA GAT GGT GAT GGG ATT TC-3'), hIRF7 (5'-TAT ACC ATC TAC CTG GGC TTC GG-3' 5'-GCT CCA GCT TTC TGG AGT TCT CAT T-3'), hOASL (5'-AGG ACT GTT GCT ATG ACA ACA GGG A-3' 5'-TGC TGC TGA GAA GCT GCC TCT C-3'), hISG15 (5'-TTT GCC AGT ACA GGA CTT GTG-3' 5'-GCT CAG AGG TTC GTC GCA TTT-3'), hIFI44 (5'-TTC GAT GCG AAG ATT CAC TG-3' 5'-CCC TTG GAA AAC AGA CCT CA-3'), mGAPDH (5'-GGT GCT GAG TAT GTC GTG GAG TCT A-3' 5'-AAA GTT GTC ATG GAT GAC CTT GG-3'), mLy6a (5'-GGA GGC AGC AGT TAT TGT GGA TTC T-3' 5'-GTG GGA ACA TTG CAG GAC CCC A-3'), mOasl2 (5'-AGC CGT GAT GGA GCT CCT CGT-3' 5'-GGA TGA TGG GCC GGT CTC CCT-3'), mOasl1 (5'-TGG AGG GTG AAG AGA GCA CCC G-3' 5'-AGG CGA GCG TGC AAT GGC TT-3'), mIrf7 (5'-TCC GGT ACC AGG GTC CAG CC-3' 5'-CGG GAG CGC ACA CGT GAT GT-3'), mIfi44(5'-TGT AAG GCT TCT GAG CAG GTT CTG A-3' 5'-TGT GGG CCT GAA CTC TGT GGG T-3').

**Replating**

After bone marrow and fetal liver cells were infected, they were cultured in 2x BM with 2 ug/mL puromycin for two days. Cells were resuspended at a density of 4 x $10^4$ cells/mL M3434 semisolid media with 1 ug/mL puromycin and 1% penicillin/streptomycin. The solution was vortexed and passed through an 18

½ gauge needle onto 30 mm wide tissue culture plates and placed in a 37° C incubator with 5% carbon dioxide. After one week, the first replating was performed. 2 mL pre-warmed IMDM was used to dissolve the M3434 media. The cells were plated out at 2 x $10^4$cells/mL of M3434 media with 1% penicillin/streptomycin but without puromycin. After one week the number of colonies was counted. The cells for each condition were counted and the cells were replated again at 2 x $10^4$ cells/mL of M3434 media with 1% penicillin/streptomycin but without puromycin.

**Bone Marrow Transplants**

Fetal liver cells (FLC) were harvested and infected as described above. C57BL/6 wild type and Ifnar1KO mice were irradiated at 700 rad. FLC were injected into the tail veins of recipient mice with a 25 gauge needle. Recipient mice were given acid treated water for three weeks following the bone marrow transplant.

**MTS Assay**

U937T, U937T-AE and U937T-AE9a cells were seeded out in duplicate into a 96 well plate at a density of 250,000 cells/mL in 100 µL. Cells were treated with MTS reagent and the absorbance at 490 nm and 700 nm was measured 100 minutes after addition of the reagent. The readings of 700 nm and the readings of the blanks were subtracted from the readings of the converted MTS reagent at 490 nm to determine the actual absorbance.

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