UNIVERSITY OF CALIFORNIA, SAN DIEGO

Loss of Usp18 Impairs the Propagation of Myeloid Leukemia Cells in Mouse Models

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Yue Zhang

Committee in Charge:

Professor Dong-Er Zhang, Chair
Professor Geng-sheng Feng, Co-Chair
Professor Li-fan, Lu

2017

The thesis of Yue Zhang 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

2017

DEDICATION

I dedicate my Thesis to my parents, Robert and Rebecca, who taught me the importance of tenacity in success.

EPIGRAPH

“It takes more than one cold day for a river to freeze three feet deep.”

Chinese Proverb

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SUPPLEMENTAL FIGURES

Appendix I: Acknowledgements

First, I would like to thank Dr. Dong-Er Zhang for welcoming me, who does not have any ideas about lab work, jointing the lab. I am thankful for having a wonderful PI as my advice and guidance.

I would like to thank Kei-ichiro Arimoto as my mentor in the lab. He teaches how to work and think as a scientist with lots of patient and kindness. He gives me lots of support and guidance into the future scientific world. I also want to thank Sheila Tran, who teaches me many basic lab techniques when I just start in the lab. Least but last, I would like to thank all of the members of the Zhang lab, for each of them provided me with support.

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Chapter 2, in full, is currently being prepared for submission for publication of material. The authors are Arimoto, Kei-ichro; Zhang, Yue; Ishida, Sayuri; Zhang, Dong-Er. The thesis author was the secondary investigator and author of this paper.

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ABSTRACT OF THE THESIS

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by

Yue Zhang

Master of Science in Biology

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Professor Dong-Er Zhang, Chair

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Acute Myloid Leukemia (AML) is one of the most common leukemia in adults with poor five years survival even with modern chemotherapy treatment. New strategies need to be developed in order to enhance the survival of AML

patients. Interferon-α (IFN-α), a type I IFN, is a known as an anti-tumor agent, which used to clinical treat AML because of its anti-proliferative effect and immune response. However, the clinical trials show divergent results because of the instability and delivery difficulty. USP18 is a negative regulator of Type I IFN signaling pathway. Knockout Usp18 can enhance and prolong the Type I IFN pathway.

Here, we use AE9a-included leukemia mouse models that reflect the genetics and pathology of human acute myeloid leukemia to study the effect of *Usp18* loss in leukemia development and to discover possible new therapeutic targets for leukemia. Results show that deletion of *Usp18* delayed leukemia propagation in both the AE9a-induced and MLL-AF9-induced leukemia models. *Usp18*-deletion-mediated inhibition of myeloid leukemia propagation is IFN response dependent. More important, *Usp18* deletion can delay the myeloid leukemia remission after the chemotherapy. These data indicate that pharmacological inhibition of Usp18 may be benefit in AML treatment with interferon prescribed.

Chapter I: Introduction

Leukemia

Leukemia is one of the most common cancers in the United States (U.S.), with about 62130 new patients diagnosed and over 24,500 diagnosed patients died every year (National Cancer Institution, 2017). Leukemia is defined by the production of large numbers of abnormal blood cells in the bone marrow. The accumulation of abnormal blood cells will prevent production and function of normal hematopoietic stem cells (HSCs) (Rubnitz et al. 2010). Based on progression time and cell-type of origin, leukemia is generally classified into four major types: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoblastic leukemia (CLL), and chronic myeloid leukemia (CML).

Acute Myeloid Leukemia (AML)

Acute Myeloid Leukemia (AML) is the most common acute leukemia in elderly with median 68 years old at diagnosis (NCI). In 2017, an estimated 21,380 new cases were diagnosed ,and over 10,000 patients died in 2017 (NCI). The five years percent surviving is only 26.9% from 2007-2013 (NCI). The pathogenesis of AML involves the abnormal proliferation and differentiation of a clonal population of myeloid stem cells (De Kouchkovsky et al. 2016). Recently, European LeukemiaNet (ELN) updated guidelines on classification of AML, which takes recommendations for diagnosis and treatment of current AML patients. The updated recommendations include revised old ELN genetic categories,

proposed treatments, and clinical trials categories based on current studies (Döhner H, et al. 2017).

Similar to many other cancers, genetic abnormalities are correlated with AML, especially chromosomal translocations. One of the most common translocations in AML is the 8q22;21q22 translocation [t(8;21)], which accounts for 40% cases of FAB M2 subtype leukemia (Rowley et al. 1973; Groupe Francais, 1990). t(8,21) results in the formation of an oncogenic fusion protein, which contains *AML1*(*RUNX1*) gene on chromosome 21 and the *ETO* (*MTG8*, *RUNXIT1*) gene on chromosome 8 (Lam, et al. 2012). While AML1-ETO can promote stem cell self-renew and block hematopoietic differentiation, it is not sufficient to induce leukemia development on its own (Yuan et al. 2001). AML1-ETO requires additional mutations in order to develop leukemia. Our lab previously identified an alternatively spliced variant of AML1-ETO, AML1-ETO9a (AE9a), which can rapidly promote leukemia development in mice (Yan et al. 2004). In our studies, we mainly utilize this AE9a mouse model to investigate the effects of novel genetic perturbations and related drug treatment strategies in leukemia cells.

Type I Interferon

Interferons (IFNs) are a group of signaling proteins, which were produced by host cells in response to pathogens to activate immune response. There are three classes of IFNs: Type I IFNs, Type II IFNs, and Type III IFNs (Ivashkiv LB. et al. 2015) Among the different types of IFN, Type I IFNs were well recognized and studied. Upon release of interferon α or β, the interferon-α receptor (IFNAR,

which is composed of the IFNAR1 and IFNAR2 subunits) will activate Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which will recruit signal transducer and activator of transcription proteins (STAT). The interferon-stimulated gene factor 3 (ISGF3) complex - STAT1, STAT2 and IFN-regulatory factor 9 (IRF9) – will enter nucleus and stimulate IFN-stimulated response element (ISRE) region to turn on IFN inducible genes expression (Ivashkiv LB. et al. 2015).

Type I interferon (IFN) signaling has been a hot topic for decades. Interferon α (IFNα) has been used in clinical studies in AML since 1966 (Anguille et. al. 2011). IFNα may have three possible clinical uses: preventing AML remission, treatment for relapses after hematopoietic stem cell transplantation (HSCT) and to prevent recurrence (Smits et al. 2013). Past clinical outcomes varies between patients, and this is one of the reasons IFNα did not become a standard treatment for AML patients (Smits et al. 2013).

Type I IFN (IFNα or IFNβ) has a complex role in modulating the immune system against cancer through IFN-stimulated genes (ISGs) (Schneider et al. 2014). Recent studies have shown that Type I IFNs have an important role in the immune system against viral infection and tumor development (Ivashkiv et al. 2014; Schneider et al. 2014). They can activate antitumor immunity through stimulation of both innate and adaptive cytotoxic lymphocyte cells. Type I IFNs can also inhibit tumor growth directly by inhibiting proliferation, enhancing apoptosis, and affecting differentiation and migration of the tumor cells (Schneider et al. 2014). Other studies show that IFNs are essential for anticancer

therapies, such as conventional chemotherapies, targeted anticancer treatment, radiotherapy, and immunotherapy (Ivashkiv et al. 2014; Sistigu et al. 2014; Arimoto et al. 2017).

However, there are limitations for IFN treatments in AML patients. First, high levels of IFNs are needed to achieve a functional response, which can cause severe irreversible side effects in some patients that sometime are life-threatening (Dusheiko et. al. 1997). Even though IFN treatment can have a great impact in anticancer treatment, further studies need to be done in order to improve treatment outcomes.

Ubiquitin-Specific Protease 18 (USP18)

Ubiquitin-specific protease 18 (USP18) was first identified in AML-ETO leukemia cells (Liu et al, 1999). It belongs to a large family of proteins called ubiquitin-specific proteases (USPs), which function is to remove ubiquitin from various proteins (Hochstrasser M. 1996). USPs major functions are related to control protein degradation and signaling. One signaling function of USP18 is to inhibit type I and type III interferon signaling pathways (Francois-Newton et al. 2011, Burkart et al. 2013). *USP18* gene expression can be upregulated by type I and type III IFNs, lipopolysaccharide (LPS), and tumor necrosis factor alpha (TNF-α) (Honke et al. 2016). Deletion of USP18 would strengthen type I and type III IFNs signaling by prolonging JAK-STAT signaling (Honke et al. 2016).

Recent studies have shown that USP18 may play a role in tumorigenesis. In our lab, Yan et al. found that BCR-ABL expressing *Usp18*$^{-/-}$ bone marrow

transplantation has a prolonged latency period in CML-like myeloproliferative disease (Yan et al. 2007). Deletion of USP18 has also been shown to induce apoptosis in breast cancer cell line MCF-7 and glioblastoma cells due to the lack of USP18-negative effective on type 1 IFN signaling (Honke et. al. 2016). Lack of Usp can enhance the antitumor environment in PyVmT model of breast cancer (Burkart C. et al. 2013)

Expression of AE9a in mouse bone marrow cells is sufficient for acute myeloid leukemia, characterized by splenomegaly and high white blood cell counts. Given the inhibition function of Usp18 in IFN pathways and the efficacy of IFN in different cancer treatment, we investigated whether deletion of *Usp18* resulted impaired AML development in vivo. In our experiment, we use *Usp18* conditional knockout model, which we generate $Usp18^{flox/flox}Ubc^{ER}\text{-}Cre^{+}$. Upon tamoxifen injection, active CRE will enter nucleus to active flow to loop out exon 2 and 3 of *Usp18*, leading to deletion of *Usp18*.

Here, we report that, compared with oil controls, after transplantation of AE9a–expressing $Usp18^{flox/flox}Ubc^{ER}\text{-}Cre^{+}$ bone marrow cells into mice with 5-tamoxifen treatments result in a greatly prolonged survival rate and impairs leukemia development. This effect in leukemia development and prorogation is dependent on IFN signaling pathway. Furthermore, compare to only chemo treatment, the combination of chemo and loss of Usp18 shows greatly enhancing survival curve. This shows Usp18 could be a potent therapeutic treatment to treat cancer.

Chapter II. Conditional deletion of *Usp18* inhibits AE9a or MLL-AF9 induced leukemia progression *in vivo*

Previously, our lab has shown the AE9a-induced AML development was delayed in *Usp18* knockout mice. To determine the role of *Usp18* in leukemogenesis, we generated an *Usp18* conditional-knockout mouse model – *Usp18*$^{flox/flox}$*Ubc*^{ER}*Cre*$^{+}$ (Sup. Fig. 1a). We transduced Cre-ER-expressing *Usp18*-floxed fetal liver cells with AE9a-expressing retrovirus vectors and transplanted them into lethally irradiated recipient mice. By using expression of a CreER recombinase with its tamoxifen-dependent activity, we can control the time and tissue specific gene knockout by tamoxifen, which is metablized to 4-hydroxytamoxifen (OHT) (Feil S et al. 2009, Sup. Fig. 1b). Meanwhile, as controls, Usp18$^{+/+}$ Ubc^{ER}-Cre^{+} and Usp18$^{flox/flox}$ Ubc^{ER}-Cre^{-} were also established. GFP^{+} cells in the peripheral blood were monitored. About eight weeks after the transplantation, approximately 5% of GFP^{+} cells were present in the peripheral blood. A few weeks later, the mice eventually displayed the signs of disease (anemia, weight loss and reduced motility) and flow cytometry analyses showed that the frequency of GFP+ leukemic blasts in the spleen usually reached above 70% before mice were euthanized (data not shown). Then, the splenocytes were transplanted into sub-lethally irradiated recipient mice (Fig. 1A). The GFP+ cell frequencies and peripheral blood count were analyzed weekly. The recipient mice usually showed signs of sickness starting week 2. The recipient mice were randomly divided into two groups (n=7 per

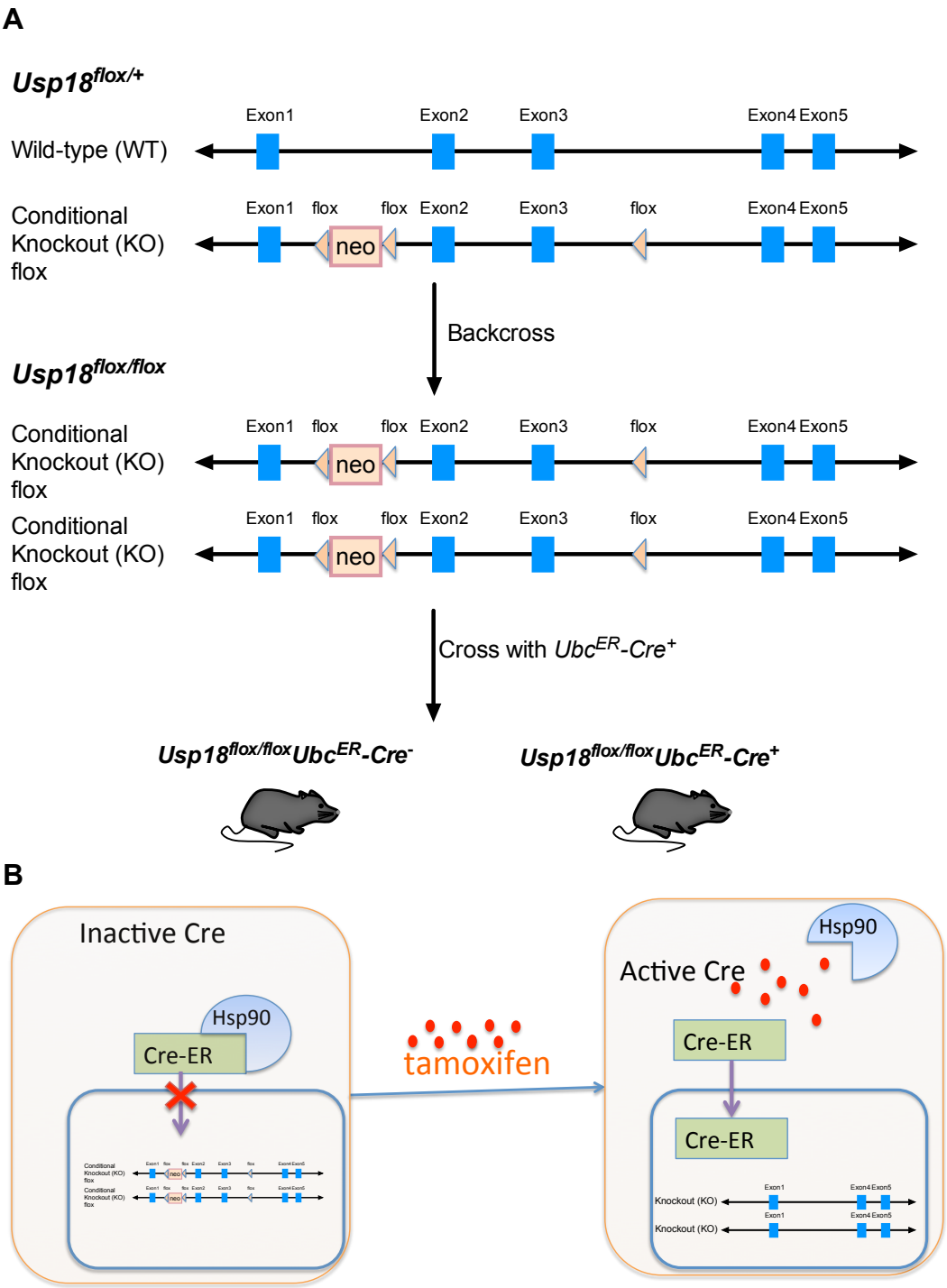
group) and treated with oil or tamoxifen. The median survival time of the mice treated with oil was 24 days (range, 22-30 days), whereas it was 40 days (range, 24-48 days) for the mice treated with tamoxifen. The survival of AE9a AML-bearing mice, although shorter than the primary leukemia mice, was significantly prolonged by treatment with tamoxifen, compared with the oil-treated mice (Fig. 1C). Similar procedures were experimented in control groups: AE9a–*Usp18*$^{flox/flox}$*Ubc*^{ER}*-Cre*$^{-}$ and AE9a–*Usp*$^{+/+}$*Ubc*^{ER}*-Cre*$^{+}$, there were no significant differences between the oil and tamoxifen treated groups in both control groups (Fig. 1B and Sup. Fig.1).

Flow cytometry analyses showed that 60-70% of cells were positive for GFP and the white blood cells (WBC) count reached up to 150x10^{3}/ml in PB before the treatment (Fig. C-D). We used flow cytometry to compare the peripheral blood of the mice transplanted with *Usp18* $^{flox/flox}$ *Ubc*^{ER}*-Cre*$^{+}$ AE9a leukemia cells and either treated with tamoxifen or oil control and found far fewer GFP^{+} cells in the tamoxifen treated mice (p=0.01, Fig. C). The WBC count also showed a significant decrease after the tamoxifen treatment compared to the control (p=0.01, Fig. D). There were less leukemia blast cells found in the peripheral blood of AE9a mice in the tamoxifen treated group compared to the oil-treated group (Fig. 1E).

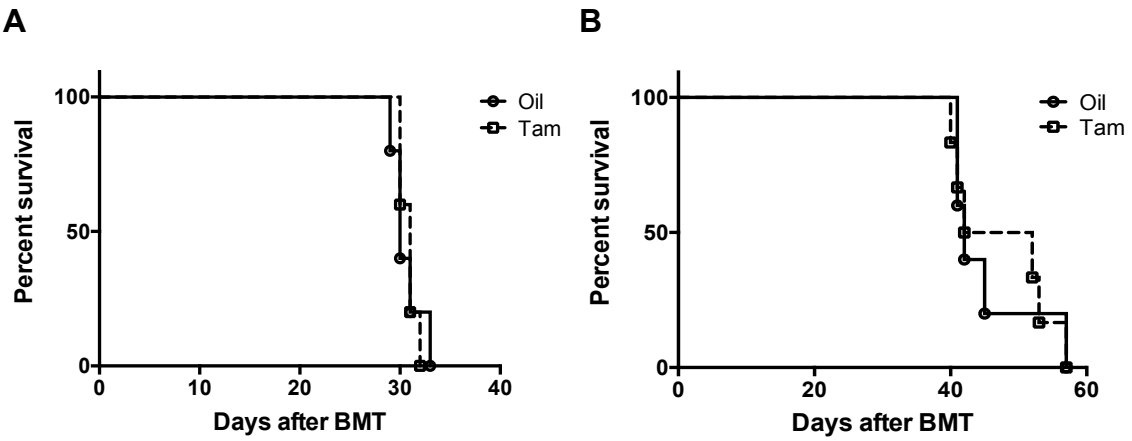
We also tested the conditional *Usp18* knockout model in an MLL-AF9 induced leukemia mouse model. We transduced Cre-ER expressing *Usp18*-floxed fetal liver cells with MLL-AF9-expressing retrovirus vectors and

transplanted them into lethally-irradiated recipient mice. The difference in overall survival was significant between the oil and tamoxifen treated groups (Fig. 1G). These results indicate that loss of *Usp18* can inhibit AE9a-induced leukemia development, establishing USP18 as a potential target for new treatment strategies in human AML.

Chapter 2, in full, is currently being prepared for submission for publication of material. The authors are Arimoto, Kei-ichro; Zhang, Yue; Ishida, Sayuri; Zhang, Dong-Er. The thesis author was the secondary investigator and author of this paper.



Supplement Figure 1. Conditional *Usp18 Knockout Strategy*
(A) Scheme of generate conditional knockout *Usp18*$^{flox/flox}$*Ubc*^{ER}*Cre*$^{+}$.
(B) Scheme of Cre-flox system.



Supplement Figure 2. Supplemental data for leukemia analysis
(A) *Usp18*$^{flox/flox}$*Ubc*^{ER}*-Cre*$^{-}$ AML1/ETO 9a GFP splenocytes from primary bone marrow transplanted mice were secondary transplanted to sub-lethal irradiated recipient mice. After the sign of the disease burden, 5 times of oil or tamoxifen was injected. Kaplan-Meier survival curve was analyzed (n=5). P-value was determined by Log-Rank (Mantel-Cox) test, p-value is not significant.
(B) *Usp18*$^{+/+}$*Ubc*^{ER}*-Cre* positive AML1/ETO 9a GFP splenocytes from primary bone marrow transplanted mice were secondary transplanted to sub-lethal irradiated recipient mice. After the sign of the disease burden, 5 times of oil or tamoxifen was injected. Kaplan-Meier survival curve was analyzed (n=5). P-value was determined by Log-Rank (Mantel-Cox) test, p-value is not significant.

Figure 1. Conditional deletion of *Usp18* inhibits AE9a or MLL-AF9 induced leukemia progression *in vivo*

(A) Schematic of AE9a - induced leukemia mouse. *Usp18*$^{+/+}$*Ubc*^{ER}*-Cre*$^{+}$, *Usp18*$^{flox/flox}$ *UBC*^{ER}*-Cre*$^{-}$ or *Usp18*$^{flox/flox}$ *Ubc*^{ER}*-Cre*$^{+}$ expressing AE9a splenocytes from primary bone marrow transplanted mice were secondary transplanted to sub-lethal irradiated recipient mice. After the first sign of leukemia, five times oil or tamoxifen (i.p.) were injected in five consecutive days.

(B) Kaplan-Meier survival curve was analyzed for AE9a-expressing *Usp18*$^{f/f}$ *Ubc*^{ER}*-Cre*$^{+}$ splenocytes secondary transplantation (n=7). P-value was determined by Log-Rank (Mantel-Cox) test, p=0.003.

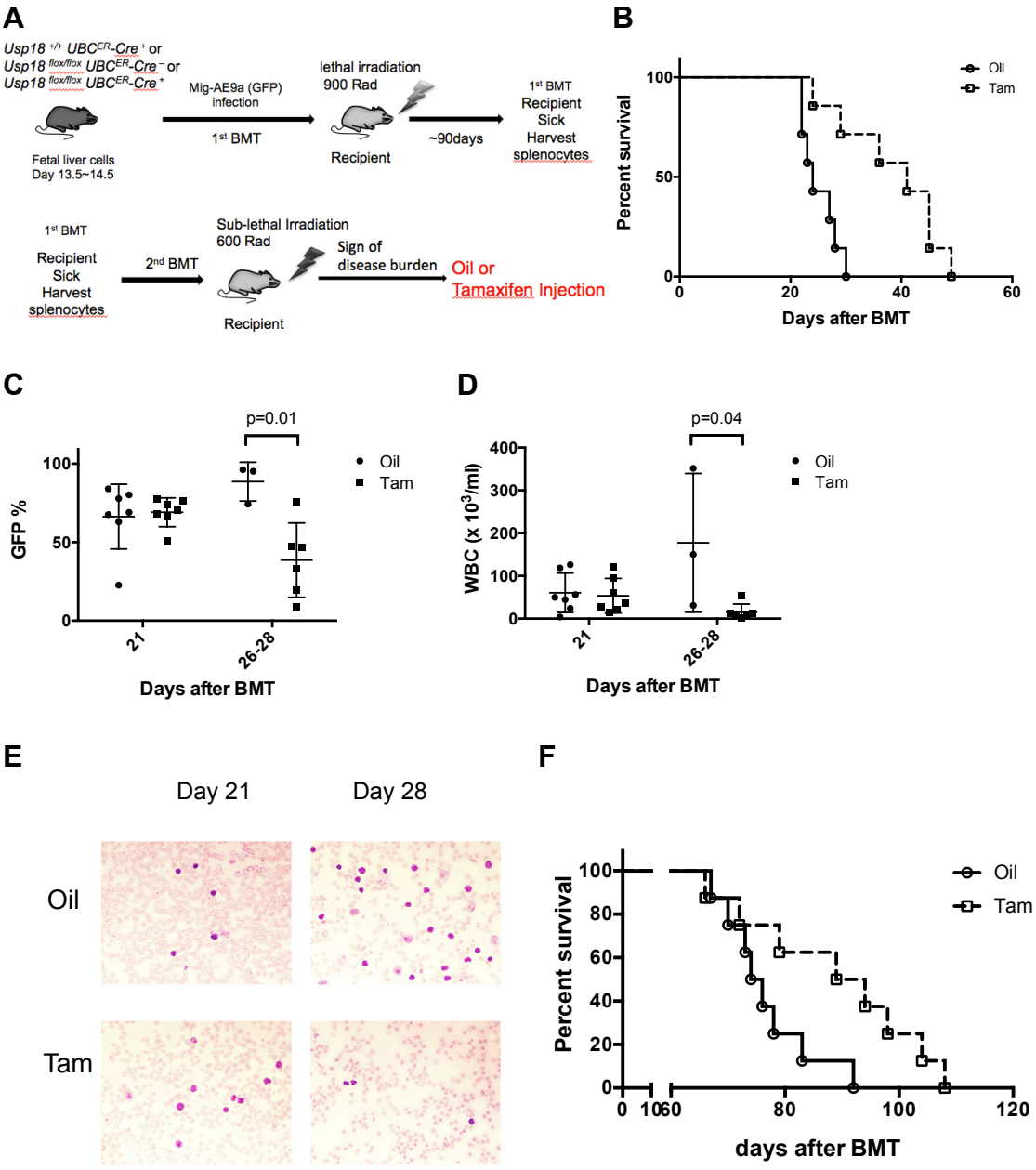
(C-E) Data for AE9a-expressing *Usp18*$^{f/f}$ *UBC*^{ER}*-Cre*$^{+}$ splenocytes secondary transplantation (n=7)

(C) GFP percent from peripheral blood (PB) was analyzed before and after the oil or tamoxifen treatment by FACS. P-value was determined by student t-test (p=0.01).

(D) White Blood Cells (WBC) from PB were analyzed before and after the oil or tamoxifen treatment by HemaVet. P-value was determined by student t-test (p=0.03).

(E) Representative blood smear before and after the oil or tamoxifen treatment.

(F)MLL-AF9 GFP infected *Usp18* $^{f/f}$ *UBC*^{ER}*-Cre* positive bone marrow cells were transplanted to lethally irradiated recipient mice. 10 times of oil or tamoxifen (i.p.) were injected staring day 36. Survival were analyzed (n = 7 each). p-value was determined by log-rank test.


A
Usp18 +/+ UBC^ER-Cre+ or
Usp18 flox/flox UBC^ER-Cre− or
Usp18 flox/flox UBC^ER-Cre+
Fetal liver cells
Day 13.5~14.5
Mig-AE9a (GFP)
infection
1st BMT
lethal irradiation
900 Rad
Recipient
~90days
1st BMT
Recipient
Sick
Harvest
splenocytes
1st BMT
Recipient
Sick
Harvest
splenocytes
2nd BMT
Sub-lethal Irradiation
600 Rad
Recipient
Sign of
disease burden
Oil or
Tamaxifen Injection
B
Percent survival
Days after BMT
Oil
Tam
C
p=0.01
GFP %
Days after BMT
Oil
Tam
D
p=0.04
WBC (x 10^3/ml)
Days after BMT
Oil
Tam
E
Day 21
Day 28
Oil
Tam
F
Percent survival
days after BMT
Oil
Tam

Chapter III. The impairing effect of loss of Usp18 in the propagation of myeloid leukemia in mouse models can be enhanced with IFN inducer

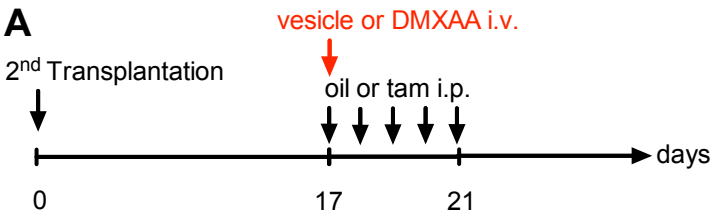
Previous studies have shown that Usp18 has two main functions, one of which is to inhibit the type I IFN signaling pathway. In order to understand how loss of Usp18 affects the leukemia development in mouse model, we used DMXAA (5,6-dimethylxanthenone-4-acetic acid, also called Vadimazan) to induce the type I IFN signaling pathway. DMXAA is a potent tumor vascular disrupting agent in mice and a potent inducer of IFN β (Jassar et al. 2005; Roberts et al. 2007).

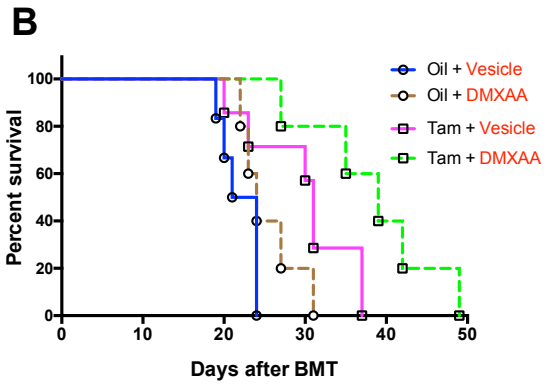
The secondary BMT AE9a induced leukemia experiment setup was similar to Fig. 1A. At day 18, the GFP+ cell frequency and WBC count were measured in PB and mice were randomly divided into four groups (n=6 per group) and received treatment starting on day 18 (Fig. 2A). As expected, mice receiving oil and vehicle treatments had the worst survival rate and the tamoxifen + DMXAA treatment had the best survival rate. The difference between the groups was significant ($p<0.01$) and overall survival was enhanced (tam + DMXAA 39d to tam + vehicle 31d, $p = 0.0578$; tam + DMXAA 39d to oil + DMXAA, $p = 0.0088$; and tam + DMXAA 39d to oil + vehicle 24d, $p= 0.0024$) (Fig. 2B). There was no significant difference between the oil and the oil + DMXAA group, which indicates that the low dosage of DMXAA we used is not sufficient to suppress the development of the leukemia on its own.

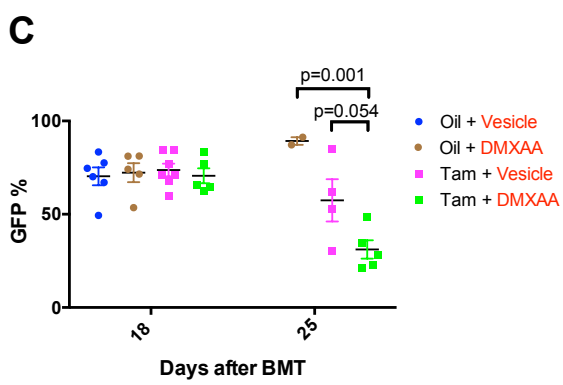
We compared the peripheral blood of the mice transplanted with *Usp18*$^{flox/flox}$ *Ubc*^{ER}*-Cre* AE9a leukemia cells in the four different combinations of treatments, by flow cytometry, and found far fewer percent of GFP$^+$ cells in the tamoxifen and DMXAA treated mice than that of other groups (Fig. 2C). The oil and vehicle treated mice were scarified before the last treatment due to advanced signs of disease. The GFP$^+$ Gr-1$^+$ CD11b$^+$ mature myeloid cell frequency was increased in the tamoxifen-treated groups. This showed that loss of *Usp18* pushed the leukemia blast cells to a more differentiated state (Fig. 2D). The WBC count showed significant decrease after the tamoxifen +DMXAA treatments compared to other groups ($p<0.001$, Fig. 2E). The mice with tamoxifen and DMXAA treatment showed less anemia and higher RBC in the four groups (Fig. 2F). These results indicate that the inhibitory effect of Usp18 deletion in AE9a-induced leukemia development can be enhanced with other IFN-inducing reagents.

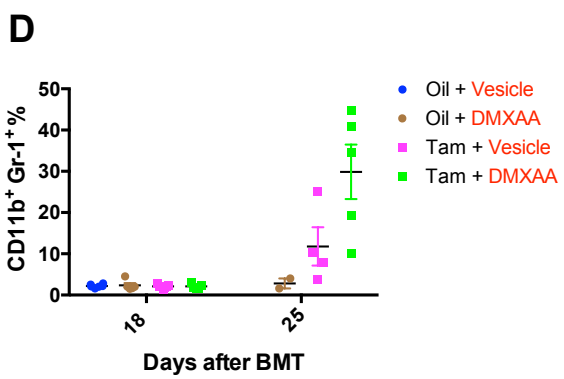
Chapter 3, in full, is currently being prepared for submission for publication of material. The authors are Arimoto, Kei-ichro; Zhang, Yue; Ishida, Sayuri; Zhang, Dong-Er. The thesis author was the secondary investigator and author of this paper.

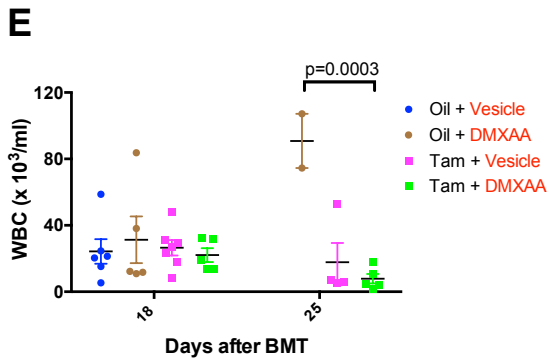
Figure 2. The impairing effect of loss of Usp18 in the propagation of myeloid leukemia in mouse models can be enhanced with IFN inducer.
(A) In vivo treatment regimen. *Usp18*$^{f/f}$ *UBC*^{ER}*-Cre*$^{+}$ AE9a GFP splenocytes from primary bone marrow transplanted mice were secondary transplanted to sub-lethal irradiated recipient mice. After the first sign of disease burden, 5 times of oil or tamoxifen (i.p.) with once vehicle or DMXAA (i.v.) were injected.
(B) Kaplan-Meier survival curve was analyzed (n=6) for *Usp18*$^{f/f}$ *UBC*^{ER}*-Cre*$^{+}$. P-value was determined by Log-Rank (Mantel-Cox) test, (oil + vesicle vs. tam + DMXAA, p=0.0024.; oil + DMXAA vs. tam + DMXAA, p=0.0088; tam + vesicle vs. tam + DMXAA, p = 0.0578).
(C–G) PB was analyzed at day 18 and 1 week after treatment day 24.
(C) GFP percent in PB was analyzed by FACS. P-value was determined by student t-test (oil + DMXAA vs. tam + DMXAA, p=0.0008; tam + vesicle vs tam + DMXAA, p=0.0543).
(D) CD11b^{+}Gr-1^{+} percent of GFP^{+} in PB was analyzed by FACS.
(E) WBC count was analyzed by HemaVet. P-value was determined by student t-test (oil + DMXAA vs. tam + DMXAA, p = 0.0003).
(F) RBC count was analyzed by HemaVet.

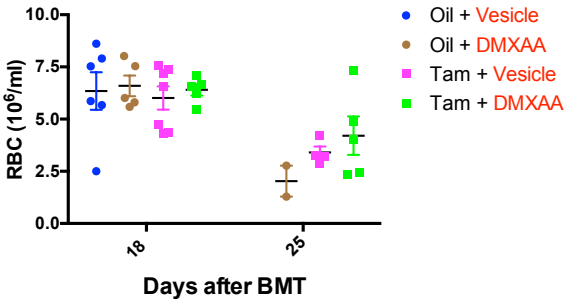

A
vesicle or DMXAA i.v.
2nd Transplantation
oil or tam i.p.
days
0
17
21


B
Oil + Vesicle
Oil + DMXAA
Tam + Vesicle
Tam + DMXAA
Percent survival
Days after BMT


C
p=0.001
p=0.054
GFP %
Days after BMT
Oil + Vesicle
Oil + DMXAA
Tam + Vesicle
Tam + DMXAA


D
CD11b+ Gr-1+ %
Days after BMT
Oil + Vesicle
Oil + DMXAA
Tam + Vesicle
Tam + DMXAA


E
p=0.0003
WBC (x 10³/ml)
Days after BMT
Oil + Vesicle
Oil + DMXAA
Tam + Vesicle
Tam + DMXAA


F
RBC (10⁶/ml)
Days after BMT
Oil + Vesicle
Oil + DMXAA
Tam + Vesicle
Tam + DMXAA

Chapter IV. *Usp18* deletion-mediated inhibition of myeloid leukemia propagation is IFN response dependent

The above results revealed that *Usp18* deletion enhanced the survival of leukemic mice when combined with an inducer of IFN. We hypothesized that the effect of *Usp18* in AE9a induced leukemia is IFN response dependent. In order to determine whether the effect of *Usp18* is dependent on the IFN response or not, we used an IFNAR1 antibody to block the Type I IFN pathway.

The secondary BMT AE9a induced leukemia experiment setup was similar to Fig. 1. At day 18, the GFP cell frequency and WBC count were measured in PB and randomly divide the recipient mice into four groups (n=6 per group) and receive treatment starting on day 18 (Fig. 3A). Mice receiving the oil + anti-IFNAR1 treatment had the worst survival rate and the tamoxifen + control-IgG1 treatment had the best survival rate. The difference between the groups was significant ($p<0.01$) (Fig. 2B). No significant difference between the oil + control IgG1 and the oil anti-IFNAR1 shows that the low dosage of anti-IFNAR1 did not effect the survival. Comparing to the oil groups, there was significant difference between the tam + control IgG1 and the tam + anti-IFNAR1, which showed that *Usp18* deletion mediated inhibition of AE9a leukemia propagation is through Type I IFN pathway ($p=0.04$, Fig. 3B).

We compared the peripheral blood of the mice transplanted with *Usp*$^{flox/flox}$ *Ubc*^{ER}*-Cre*$^{+}$ AE9a leukemia cells in the four different combination of treatments,

by flow cytometry, and found far fewer GFP^+ cells in the tamoxifen + control-IgG1 than the tamoxifen + anti-IFNAR1 treated mice (Fig. 3C). The oil + anti-IFNAR1 treated mice were sacrificed due to severe disease progression. The GFP^+ $Gr\text{-}1^+$ $CD11b^+$ cell frequency was decreased in the tamoxifen + anti-IFNAR1 treated group compared to tamoxifen treatment. This showed that blocking IFN pathways could mimic the effect of loss of *Usp18* in leukemia blast cells and result in increased differentiation (Fig. 3D). The WBC and RBC counts showed similar results that tamoxifen with anti-IFNAR-1 had worse disease condition than tamoxifen only treatment (Fig. 3 E-F). These results indicate that inhibition effect of Usp18 deletion in AE9a induced leukemia development is through Type I IFN signaling pathway.

Chapter 4, in full, is currently being prepared for submission for publication of material. The authors are Arimoto, Kei-ichro; Zhang, Yue; Ishida, Sayuri; Zhang, Dong-Er. The thesis author was the secondary investigator and author of this paper.

Figure 3. *Usp18* deletion-mediated inhibition of myeloid leukemia propagation is IFN response dependent

(A) In vivo treatment regimen. *Usp18*$^{f/f}$ *UBC*^{ER}*-Cre*$^{+}$ AE9a GFP splenocytes from primary bone marrow transplanted mice were secondary transplanted to sub-lethal irradiated recipient mice. After the first sign of disease burden, 5 times of oil or tamoxifen (i.p.) with once control-IgG1 or anti-IFNAR1 (i.v.) were injected.

(B) Kaplan-Meier survival curve was analyzed (n=6). P-value was determined by Log-Rank (Mantel-Cox) test, (oil + control-IgG1 vs. tam + control-IgG1, p=0.0016; tam + anti-IFNAR1 vs. tam + control-IgG1, p=0.0398).

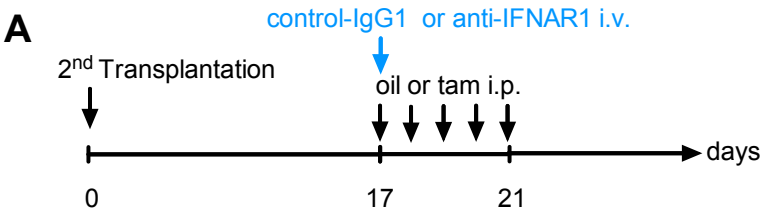
(C–G) PB was analyzed at day 18 and 1 week after treatment day 24.

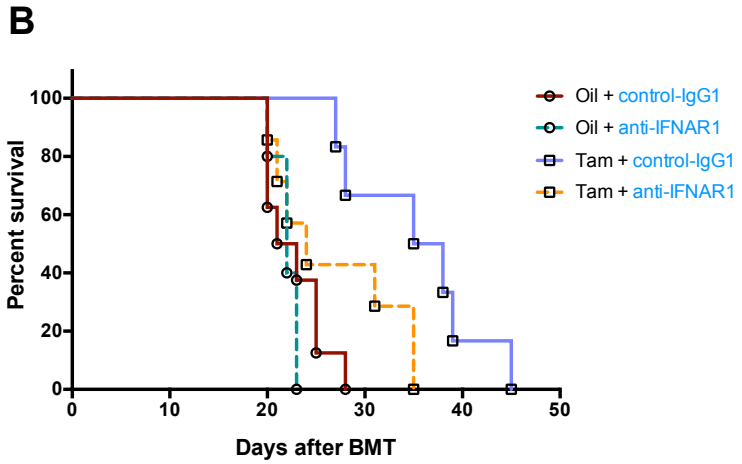
(C) GFP percent in PB was analyzed by FACS. P-value was determined by student t-test.

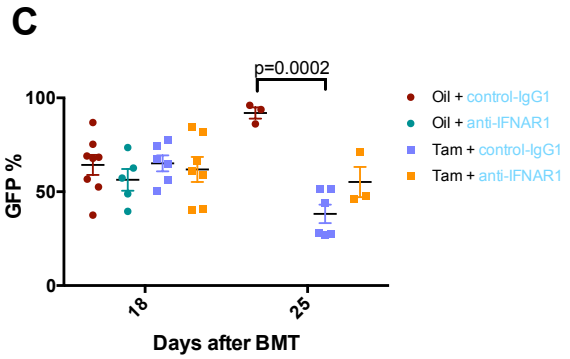
(D) CD11b^{+}Gr-1^{+} percent of GFP^{+} in PB was analyzed by FACS.

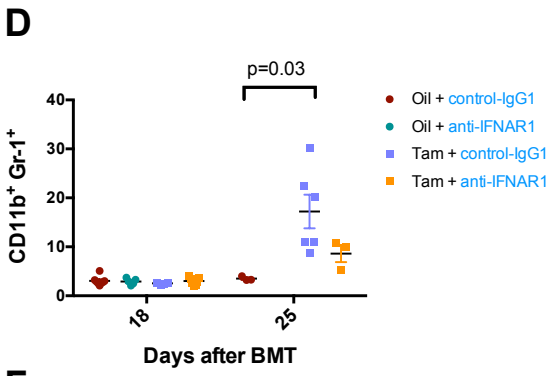
(D) WBC count was analyzed by HemaVet. P-value was determined by student t-test.

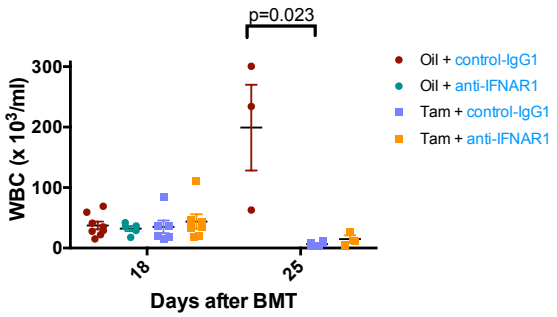
(E) RBC count was analyzed by HemaVet.

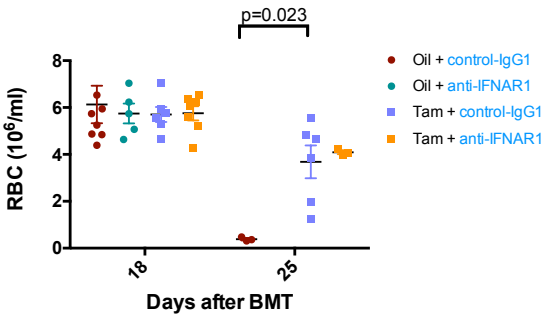

A
control-IgG1 or anti-IFNAR1 i.v.
2nd Transplantation
oil or tam i.p.
days
0
17
21


B
Percent survival
Days after BMT
Oil + control-IgG1
Oil + anti-IFNAR1
Tam + control-IgG1
Tam + anti-IFNAR1


C
p=0.0002
GFP %
Days after BMT
Oil + control-IgG1
Oil + anti-IFNAR1
Tam + control-IgG1
Tam + anti-IFNAR1


D
p=0.03
CD11b+ Gr-1+
Days after BMT
Oil + control-IgG1
Oil + anti-IFNAR1
Tam + control-IgG1
Tam + anti-IFNAR1


E
p=0.023
WBC (x 10³/ml)
Days after BMT
Oil + control-IgG1
Oil + anti-IFNAR1
Tam + control-IgG1
Tam + anti-IFNAR1


F
p=0.023
RBC (10⁶/ml)
Days after BMT
Oil + control-IgG1
Oil + anti-IFNAR1
Tam + control-IgG1
Tam + anti-IFNAR1

Chapter V. Loss of Usp18 delays remission of AE9a induced AML after chemotherapy in vivo

We used a previously established chemotherapy regimen involving intraperitoneal (i.p.) injection of cytarabine (100mg/kg per day for 5 days) and doxorubicin (3mg/kg per day for 3 days) to study the effect of Usp18 deletion. These dosages can mimic the standard induction therapy used to treat AML in human patients (Zuber et al. 2009) (Fig. 4A). To slow down the AE9a induced leukemia in mice, we transplanted less AE9a *Usp18*$^{flox/flox}$ *Ubc*^{ER}*-Cre*$^{+}$ cells compared to the previously described experiments. We compared the peripheral blood of the mice transplanted with *Usp18*$^{flox/flox}$ *Ubc*^{ER}*-Cre*$^{+}$ AE9a leukemia cells before and one week after the chemotherapy, by flow cytometry, and found that most of recipient mice's GFP^{+} cell frequency were lower than 0.8% (Fig. 4C). The chemo treated recipient mice were randomly divided into two groups and treated with oil or tamoxifen (everyday for the first 5 days, then once every 3 days) (Fig. 4A). The median survival time of the mice treated with oil was 45 days (range, 41-45 days), whereas it was 72 days (range, 64-100+ days) for the mice treated with tamoxifen. The survival of AE9a AML-bearing mice was significantly prolonged by treatment with tamoxifen, compared with the oil treated mice ($p<0.01$)(Fig. 4B). The remission of oil treated group came back four weeks after BMT; whereas, remission of tamoxifen treated group did not come back until six weeks and remain low percent of GFP^{+} until eight weeks after BMT (Fig. 4C).

These results indicate that loss of *Usp18* delays remission of AE9a induced AML after chemotherapy.

Chapter 5, in patial, is currently being prepared for submission for publication of material. The authors are Arimoto, Kei-ichro; Zhang, Yue; Ishida, Sayuri; Zhang, Dong-Er. The thesis author was the secondary investigator and author of this paper.

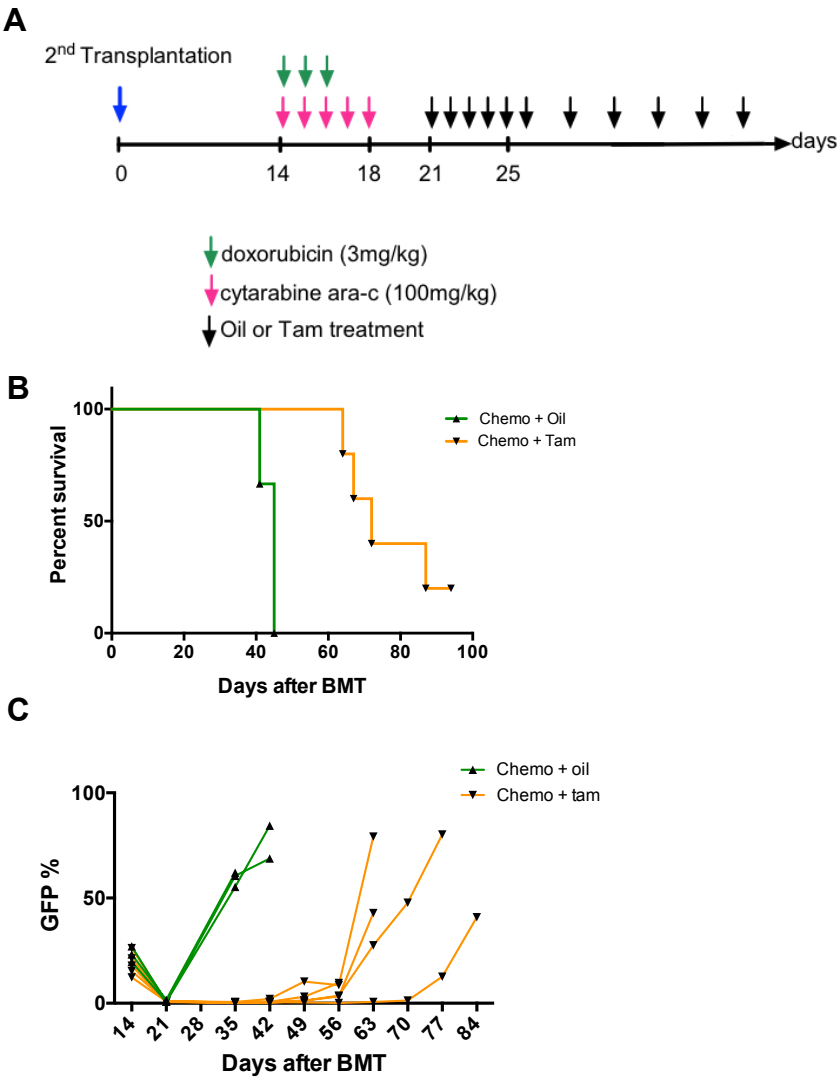


Figure 4. Loss of Usp18 delays remission of AE9a induced AML after chemotherapy in vivo

(A) In vivo treatment regimen. *Usp18*$^{f/f}$ *UBC*^{ER}-*Cre*$^{+}$ AE9a GFP splenocytes from primary bone marrow transplanted mice were secondary transplanted to sub-lethal irradiated recipient mice.

(B) Kaplan-Meier survival curve was analyzed (n=3). P-value was determined by Log-Rank (Mantel-Cox) test p=0.0029.

(C) GFP percent in PB was analyzed by FACS weekly.

Chapter VI Discussion

In this study, we use mouse leukemia models that reflect the genetics and pathology of human acute myeloid leukemia, in order to study the effect of Usp18 loss in leukemia development and to discover possible new therapeutic targets for leukemia. We have found that loss of Usp18 delayed leukemia propagation and development in both the AE9a-induced and MLL-AF9-induced leukemia models. Our observations provide important evidences for the role of Usp18 in AE9a induced leukemia progression and suggest that deletion of Usp18 may be useful in the therapy of human leukemia with or without chemotherapy.

Interferon-α (IFN-α) is a well-known anti-tumor agent and it is used in the treatment of several solid tumors (Ferrantini M. et al. 2007). Even though there is a clear biological benefit in AML, IFN-α did not reach the expectation and failed to use to treat AML. However, as more studies are done in IFNs, new insights of IFN-α treatment in clinical efficiency and pharmaceutical improvements help to reconsider its possible treatment in AML (Benjamin R. et al. 2007; Berneman Z. et al. 2010). AML cells can respond to IFN-α, which induces apoptosis and differentiation, and inhibition of proliferation. IFN-α can also activate immune response such as DCs to increase antitumor environment. Two of the factors that affect the efficacy of IFN-α in AML clinical treatment are delivery and stability (Benjamin R. et al. 2007; Arnaud P. et al. 2002). Deletion of *USP18* can prolong

and enhance Type I IFN signaling pathway. Therefore, the inhibition of Ubp43 or other negative regulators of IFN signaling can be useful drugs to treat AML.

USP18 deletion also inhibits leukemia development even after chemotherapy remission. The chemotherapy is the primary treatment for AML. The primary refractory of AML with chemotherapy is about 40-45%, which is relatively high. Some elder patient could not go through the second cycle of high dosage of cytarabine after the primary induction failure because the physical conditions. Combination of drugs after the primary chemotherapy may decrease the rate of primary refectory, or give the possible second treatment to elder patients. Our data shows that *USP18* deletion can delay the remission after the chemotherapy, which suggests Usp18 may be a possible therapeutic target for AML.

In summary, we show that loss of *Usp18* has inhibition effect in the propagation of AML in AE9a-induced leukemia mouse model. By using an IFN inducer, loss of *Usp18* mediated enhanced survival in AE9a-induced leukemia mice is enhanced. Moreover, *Usp18*-deletion-mediated inhibition of myeloid leukemia propagation is IFN response dependent. Remarkably, *Usp18* deletion can delay the myeloid leukemia remission after the chemotherapy. Thus, targeting *Usp18* may be a potential approach to enhance the modern treatment of leukemia. Furthermore, *Usp18* may be also an approach to enhance the modern treatment to other cancers.

One important consideration:

Our conclusions are far form definitive because we do no know where the GFP+ cells in tamoxifen-treated AE9a mice are *Usp18* deleted or not when the mice shows severe sign of disease burden. Tamoxifen is effective; yet, it could not completely delete *Usp18* in all GFP^+. Some of the leukemia cells will be heterozygous for *Usp18* and that affects the leukemia cells were unknown. Based on data, there was a clear reduction of GFP+ after the tamoxifen treatment. However, the GFP+ cells came back in a very short period of time. With these in mind, we are left to discuss, is the GFP^+ cell *Usp18* deletion? If it is not, why does there is a decrease of GFP^+ after the tamoxifen treatment?

Future Directions

While these studies show that loss of Usp18 has effect in leukemia development, how well does it effect was unknown. There are many ways to continue exploring the function of USP18 in cancer and normal HSCs, as well as investigating how to knockout USP18 *in vivo*. Additional studies on the effect of loss of USP18 in both cancer and normal cells will help us to understand how to improve the possible new treatment in cancer.

In order to study the loss of Usp18 in other types of blood cancer, we would like to establish similar bone marrow transplantation experiments to study other type of leukemia. One model we choose MLL translocation, and we used the MLL-ENL to model the most common and associate with a particularly poor

prognosis (Schoch et al. 2003). MLL-ENL is associated with poor chemotherapy outcome and will be a good model to see whether loss of Usp18 will prolong the survival or not.

Another problem needed to address is how to block or delete USP18/Usp18 in human patients. First, we need do addition studies to investigation whether blocking the enzymatic activity of USP18 sufficient to mimic the effects as Usp18 knockout. We can use USP18 blocker and Usp18 deletion *in vivo* to compare the difference effect in AML. Second, we can also further investigate that whether gene therapy, such as CRISPR, can be used.

Appendix I: Materials and Methods

Animals

The C57BL/6J background $Usp18^{flox/flox}$ mice and C57BL/6J background Ubc^{ER}-Cre^{+} are crossed to generation $Usp18^{+/+}$ Ubc^{ER}-Cre^{+}, $Usp18^{+/+}$ Ubc^{ER}-Cre^{-}, $Usp18^{flox/flox}$ Ubc^{ER}-Cre^{+} and $Usp18^{flox/flox}$ Ubc^{ER}-Cre^{-} mice for BMT. Mice were housed in a pathogen-free facility. All procedures were conducted at the University of California at San Diego (UCSD), and performed as approved by the Institutional Animal Care and Use Committee and according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Murine Fetal Liver Transduction and Transplantation

Embryonic day 13.5–14.5 fetal liver cells were harvested from $Usp18^{+/+}$ Ubc^{ER}-Cre^{+} mating with $Usp18^{+/+}$ Ubc^{ER}-Cre^{-} and $Usp18^{flox/flox}$ Ubc^{ER}-Cre^{+} mating with $Usp18^{flox/flox}$ Ubc^{ER}-Cre^{-} females. PCR of fetal was preformed to identify the genotype. The cells were cultured in IDMEM media with 50 ng/mL recombinant SCF, 10 ng/mL murine IL-6, and 10 ng/mL murine IL-3. After overnight stimulation, cells were collected and transduced with two rounds of retrovirus MIGR1-AE9a. Post-transduction, cells were collected, counted, and transplanted into lethally (900 rad) irradiated PEP3 recipient mice (6-8 weeks old) by tail vein injection. Mice were maintained in sterilized cages for 3 weeks on acidified water (pH 4.0). Upon leukemia development, moribund mice were killed. The splenocytes were harvested for secondary bone marrow transplantation.

All recipient PEP3 mice (6-8 weeks old) were sub-lethally irradiated with 6Gy four hours before 2nd bone marrow transplantation. Different dosages of leukemia splenocytes from primary bone marrow transplanted mice were transplanted by tail vein injection. We sub-lethally irradiated recipient PEP3 mice (7-9week old) with 600 rad and $0.5x10^5$ total splenocytes from primary transplanted sick mice were transplanted by tail vein injection. Mice were maintained in sterilized cages and given acidified water (pH2.7).

Blood Collection and Blood Count

Peripheral blood of recipient mice was collected weekly after transplantation. The distal 3mm of the tail was clipped and micro-Hematocrit capillary tubes (Fisherbrand 22-362-566) were used to collect peripheral blood. The blood was obtained microvette containing anticoagulants (EDTA for cell counting). Immediately after collection, the cut surface of the tail was cauterized with styptic powder (Kwik-stop, ARC Labo- ratories, Atlanta, GA). A complete blood count was preformed with Hemavet Hematology System Counter (Scil Vet abc plus). Blood smear was stained by Wright and Giemsa stain.

Flow Cytometry

The percent of GFP, CD11b (PE-conjugated rat anti-mouse CD11b, BD Biosciences) and Gr-1 (PerCP-Cy™5.5-conjugated rat anti-mouse Ly-6G/Ly-6C, BD Biosciences) surface expression were performed by flow cytometry weekly.

Samples were analyzed on a BD FACS Canto equipped with standard lasers and filters.

Drug Treatment

Both corn oil (100ul/mice CAS 8001 Sigma) or Tamoxifen (2mg/mice, Sigma, T-5648) were intraperitoneally (i.p.) injected for 5 continuously day. Some groups were continuous i.p. injected of corn oil or Tamoxifen for every three days.

Vehicle (100ul/mice, 7.5% $NaHCO_{3,}$) or DMXAA (0.4mg/mice, D5817, Sigma) were intravenous injection (i.v.) injected with the first oil or tam treatment.

Control anti-mouse-IgG1 (0.25mg/mice, BE0241, BioXCell) or anti-mouse-IFNAR1 (0.25mg/mice, BE0241, BioXCell were intravenous injection (i.v.) injected with the first oil or tam treatment.

Chemotherapy was initiated upon detection of above 5% of GFP^+ by flow cytometry. Mice were treated for five consecutive days every 24 h with i.p. injections of 100 mg/kg cytarabine (Hospira, Zydus Hospira Oncology Private Ltd, India); during the first 3 d, 3 mg/kg doxorubicin (APP, Fresenius Kabi USA) was administered in a separate i.p. injection. Immediate response and long-term treatment effects were monitored weekly by flow cytometry and blood count.

Statistical Analysis

Data were presented as mean ± SD and analyzed using Prism 6 Software. The significance of differences between all groups was determined using multiple t-test. The significance of differences between two groups was determined using independent-sample t test. Survival curves were produced using the Kaplan-Meier estimation and the significance of differences was determined using the Log-Rank (Mantel-Cox) test.

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