The Pennsylvania State University

The Graduate School

Department of Veterinary and Biomedical Sciences

THE ROLE OF ENDOGENOUS LIPID MEDIATORS IN MODELS OF EXPERIMENTAL LEUKEMIA

A Dissertation in

Immunology and Infectious Diseases

by

Emily R. Finch

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

August 2016

The dissertation of Emily R. Finch was reviewed and approved* by the following:

K. Sandeep Prabhu
Professor of Immunology and Molecular Toxicology
Advisor of Dissertation
Chair of Dissertation Committee

Robert F. Paulson
Professor of Veterinary and Biomedical Sciences

Margherita T. Cantorna
Distinguished Professor of Molecular Immunology
Chair of the Graduate Program

Connie J. Rogers
Associate Professor of Nutrition

Penny M. Kris-Etherton
Distinguished Professor of Nutrition

*Signatures are on file in the Graduate School

ABSTRACT

Leukemia is defined by the cell lineage affected (myeloid or lymphoid) and the aggression of the disease (acute or chronic). Well-defined animal models of myeloid leukemia can be used to study specific chromosomal translocations, namely BCR-ABL, associated with disease. Though sophisticated targeted therapies are available for chronic myeloid leukemia (CML), and to a lesser extent acute myeloid leukemia (AML), they will never cure the disease. These therapies are the first of their kind; tyrosine kinase inhibitors specifically inhibit the constitutively activated *abl* domain in the BCR-ABL mutation (Philadelphia chromosome), which is associated with CML. In this dissertation, the mechanism through which diet inhibits leukemia stem cells (LSCs) is studied in the context of bioactive lipid reprogramming. LSCs are resistant to TKI therapy, as they do not depend on BCR-ABL signaling for survival. Dietary interventions to specifically impact the generation of endogenous bioactive lipids, namely a unique class of cyclopentenone prostaglandins (CyPGs), have proven to be a potential anti-leukemic therapy. CyPGs, including Δ^{12}-PGJ_2 and Δ^{12}-PGJ_3, are able to change the phenotype of LSCs from the generally quiescent state, to one, which is self-destructive. Without the addition of conventional therapy, diet is able to limit the progression of three models of myeloid leukemia, with the potential of translation to a clinical setting.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

IMPORTANT ABBREVIATIONS

ALA: α-linolenic acid (C18:3; n-3)

AML: acute myeloid leukemia

ARA: arachidonic acid (20:4 n6)

ATM: ataxia telangiectasia mutated

BCR-ABL: oncogene resulting from Philadelphia (Ph) chromosome (the reciprocal translocation between the long arms of chromosome 9 and 22 t(9:22)(q11))

BM: bone marrow

BMDM: bone marrow derived macrophage

CBC: complete blood count

CITED2: CBP/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2

CML: chronic myelogenous leukemia

COX (cyclooxygenase (-1/-2)): COX-1 (homeostatic) or COX-2 (inducible)

CRTH2: chemoattractant-homologous receptor expressed on Th2 cells

CSC: cancer stem cell

CyPG: cyclopentenone prostaglandin (generally referring to J series PGs)

Δ^{12}-PGJ$_2$: Delta-12 prostaglandin J2 (derived from ARA)

Δ^{12}-PGJ$_3$: Delta-12 prostaglandin J3 (Derived from EPA)

15d-PGJ$_2$ (15d-$\Delta^{12,14}$-PGJ$_2$)**:** 15-deoxy-delta-12,14 prostaglandin J2

DHA: docosahexaenoic acid (C22:6; n-3)

DP1 (Ptgdr): prostaglandin D receptor 1

EPA (20:5, n3): eicosapentaenoic acid

FACS: fluorescence-activated cell sorting

GPCR: G protein-coupled receptor

H&E: hematoxylin and eosin

HIF: hypoxia inducible factor

H-PGDS: hematopoietic prostaglandin D synthase

HSC: hematopoietic stem cell

IMDM: Iscove's Modified Dulbecco's Medium

i.p.: intra-peritoneal, in the context of drug delivery injection

LA: linoleic acid (C18:3; n-6)

LC-MS/MS: liquid chromatography-tandem mass spectrometry

L-PGDS: lipocalin-type prostaglandin D synthase

LPS: lipopolysaccharide

LSC: leukemia stem cell

LSC-CFU: leukemia stem cell-colony forming unit

i.v: intra-venous

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NSAID: non-steroidal anti-inflammatory drug

OA: oleic acid

PG: prostaglandin

PGES: prostaglandin E synthase

PLA_2: phospholipase A_2

PUFA: polyunsaturated fatty acids

15-PGDH: 15- prostaglandin dehydrogenase

PPARγ: peroxisome proliferator- activated receptor gamma 3

RBC: red blood cell

ROS: reactive oxygen species

Se-A: host fed selenium-adequate diet (0.08ppm as selenite)

Se-D: host fed selenium-deficient diet (<0.01ppm as selenite)

Se-S: host fed selenium-supplemented diet (.4 ppm as selenite)

Sec: selenocysteine

TKI: tyrosine kinase inhibitor

TrxR: thioredoxin reductase

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

TXAS: thromboxane A synthase

TZD: thiazolidinedione

WBC: white blood cell

ACKNOWLEDGEMENTS

The past five years have been the most exciting, stressful, and rewarding of my life. I have grown and matured as a person and scientist, but could not have done it without the help of those around me. I want to thank everyone who has been a part of this experience.

The ones who taught me:

I was very fortunate to find a graduate research project in the laboratory of Dr. K. Sandeep Prabhu. His love for science is contagious and he has pushed me to accomplish more than I thought possible. Sandeep is always encouraging, and expects the best. He has been a mentor, editor, mediator, and jokester.

As an undergraduate at Penn State, I worked in many nutrition laboratories. However, I was most interested in seeing how nutrition and energy relates to cancer progression. Working in Dr. Connie Rogers laboratory for the last year of undergraduate education was exactly what I was looking for. Dr. Rogers has been an important part of my development as a scientist. She has always been encouraging and willing to help. I have been fortunate to have her as a mentor and friend.

I would like to thank Dr. Robert Paulson, who has been a mentor since the beginning of my PhD work. The collaboration between the labs allowed me to understand both science, and lab dynamics, from multiple angles. Dr. Paulson is always logical and encouraging. I was introduced to Dr. Margherita Cantorna as an undergraduate when I applied to the IID program. She is an advocate for graduate students and a great person to have on your side. As a nutrition undergraduate, the research of Dr. Penny Kris-Etherton was awe-inspiring. I am so thankful to have her as a member of my committee, as she brings a very unique perspective.

The ones who helped me:

The bench-work presented in this dissertation would not have been possible without teamwork. By chapter, I would like to thank those who helped. In chapter 2, I thank Dr. Shailaja Hegde for irradiation of mice, Dr. Naveen Kaushal for assisting with bone marrow transplantation, Dr. Ramesh Ramachandran for help with microscopy, and Dr. Ruth Nissly who has FAC sorted more cells than I can count. In Chapter 3, Dr. Laura Goodfield (LG) and Laura Bennett (LB) were very helpful hands and good company on long experimental days. I would not have been able to harvest and count all the samples without the Lauras. Dr. Diwakar B. Tukaramrao ran LC-MS/MS samples, which he also helped to analyze. In Chapter 4, the work of Dr. Ujjawal Gandhi set the stage. Additionally, LG helped with basophil activation, colony assays, and in vivo experiments. In all chapters, the generation of retrovirus to establish the leukemia models would not have been possible without Mike Quickel.

The laboratory environment was also a huge factor in my ability to work. Dr. Shakira Nelson was in the lab when I first started. Shakira was (and still is!) a wonderful mentor and friend. She has been very encouraging and will always be someone I look up to. Dr. Vivek Narayan was in the lab for the first three years of my PhD work. He taught me to be a confident scientist and to ask the right questions. I am sad to be leaving the current lab members behind. But this is not good-bye, just “until next time.” Ashley Shay is a caring friend and strong scientist. Chang Liao is as sharp as she is sweet. I will always wear pink on Wednesday and think of her! Shaneice Nettleford started in the lab, as I was finishing. She has a big heart and is eager to learn!

The ones I taught:

I had barely begun my graduate studies when Nicole Wilski started her undergraduate research in the Prabhu Lab- and I was supposed to be her mentor! We grew up together in many ways. We will forever share the woes and patterns in EPA-diet feeding to mice. At the best of

times, we shared in our similar and contagious laughter! At others, we discussed the proper use of Sharpie markers… My second undergraduate, Jocelyn Stoudt, is the little sister I never had. She has a fun and loving personality and is also a dedicated learner. She helped me to re-connect with my youth and tried to make me cooler by introducing me to things like SnapChat and Instagram.

The ones who supported me:

My family deserves much of the credit for getting me through this process. I was lucky enough to live so close to home, and took full advantage of this support system! My mother, Amy Haworth, is my forever cheerleader and friend! I could write a book about all of the reasons how and when she made/makes my life better. If I could say it too many times I would: thank you, thank you. My father, Dr. Daniel Haworth, has always been my role model. He is the most levelheaded, intelligent, and caring father a girl could hope for! My sister, Erin Haworth, shares in my love of power and dogs. She always provided me with the important things, like massages and late-night talks. To my husband and best friend Benjamin G. Finch, thank you for supporting me, feeding me, loving me, and dealing with my crazy. My in-laws, Drs. Linda and Derrill Finch, are kind to the core. They have welcomed me into their family with open arms, and have been very supportive in my purist of a PhD. Last, but certainly not least, I would like to thank ALL THE DOGS! Bella is my parent's dog, but I consider her, my own. She has the energy and love of a puppy and always is there to give me kisses. Henry is my first baby. He has helped me stay sane and is the perfect distraction from all of life's woes. Discovering Karen Hack, of the 4-H department, and her Seeing Eye dog puppies (Vader, Epic, and Webb) was life-saving. On bad days (and good ones too!) visiting with Karen and her pups was the perfect stress reliever.

I am sure I have not named all of the people who have helped me. This is not meant to be an oversight. Thank you to everyone who has been part of this great adventure.

Chapter 1

Introduction

Leukemia: Past-to-Present

General Leukemia

Leukemia is a general term for a class of multiproliferative cancers that develop in the bone marrow. Healthy hematopoietic stem cells (HSCs) become genetically altered. These transformed cells (blasts) expand in the bone marrow and travel into the peripheral blood. The four common forms of leukemia are named based on the effected immune lineage and the duration it takes for the disease to progress, as follows: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoid leukemia (ALL), and chronic lymphoid leukemia (CLL) (1) (Fig 1-1). Acute leukemias occur quickly, progressing in weeks or less. Chronic leukemias take longer to progress, taking several years to progress through the chronic phase. Myeloid and lymphoid refer to the cell lineage affected. The focus of this review is on treatment of chronic myeloid-derived leukemias, with an emphasis on current therapies, including endogenous lipid mediators that act to inhibit the disease. While AML will be discussed, this is a much more complex disease as compared to CML, in term of its origin and genetic alterations (2-5).

Recorded CML cases began in the 1840's. Independent investigators observed similar symptoms, with one of the first documented cases by Dr. John H. Bennett (6). Bennett observed a blood disorder that he described as "puss in the blood," also presenting with an enlarged spleen (7). Interestingly, it was early cases of CML that led to understanding of white blood cells (WBCs) as a distinct type of circulatory cells (8).

Clinically, the progression of CML is somewhat predictable. Clonal expansion of a single cell, containing a characteristic genetic change, gives rise to the disease. The expansion of cells is associated with several mutations, including BCR-ABL. In the chronic phase, functionally

immature granulocytes are generated in excess (9). As indicated by its name, this chronic phase can be slow, with the median duration being three to four years (10). However, once developed, the disease can quickly progress to the blast crisis phase. The progression to this stage may also include additional mutations (11).

General signs and symptoms of CML include weakness, fatigue, weight loss, fever, bone pain, enlarged spleen, and increased WBCs in the periphery (12). Many of these are non-specific, which can make the diagnosis difficult. The frequency of CML is one to two instances per 100,000 people per year, with the median age for onset being 65 years or older (13). The disease is more common in men, with females also showing higher survival (12). Though the prevalence of CML is lower than other cancers, there is still a potential for better treatment strategies. Additionally, models of CML are well established and can be used to better understand other malignancies.

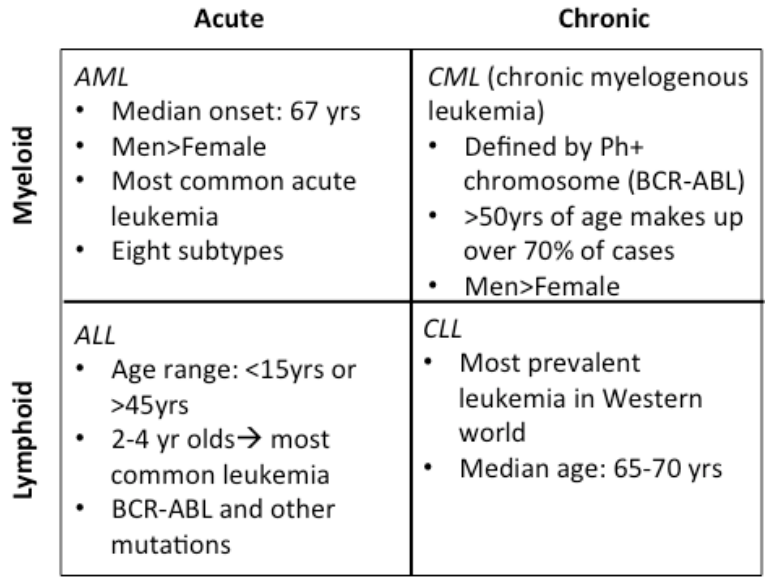

Figure **1-1**: Major forms of leukemia.

CML is the best-characterized form of leukemia. The general characteristics of the four major forms of leukemia are based on 1) progression rate (acute or chronic); 2) immune branch affected (myeloid or lymphoid); 3) age and sex; 4) specific mutations discovered.

History of CML Treatments

Throughout documented history, hematological diseases have been at the forefront of drug development (14). Treatments for these diseases, including CML, have been translated to other solid tumors with varying success. Understanding the history of drug development aids in predicting what new therapies may be successful for not only the management and elimination of CML cells, but also treatment in other cancers.

Starting in 1865, a commonly used arsenic solution (Fowler's solution; 1% trioxide) was used to treat suspected CML patients (15). Thomas Fowler introduced this arsenic solution in 1786 to treat many other aliments, including headaches and fevers. Large doses of arsenic were used in the late 19th century to treat CML. Arsenic solutions are still studied in research settings for relief of inflammation, as the mechanism of action remains unknown (6, 16). A noteworthy investigator in the early days of CML research is Dr. (Sir) Conan Doyle. In an 1882 letter to the *Lancet,* Dr. Conan Doyle described his observation of a 29-year old "well-built" man that presented with a self-described tumor in his abdomen, which had developed over several weeks. This "tumor" was the patients' enlarged spleen (6). The other major early therapy for CML was a splenectomy (from 1863-1935). In 1926, Dr. Mayo showed there was a 6.7 % decrease in mortality with a splenectomy; however, it reportedly made the patients more comfortable (6).

In 1903, targeted radiotherapy was used to reduce the size of the spleen. As demonstrated by Professor Nicholas Senn, targeted irradiation by x-ray therapy decreased the size of the spleen as well has decreased leukocytes in the periphery (6). Benzene, at a dose of four-to-five grams per day (oral or suppository) was used to treat CML from 1912-1935. After WWII, the cytotoxic properties of mustard gas were studied. There was an observation of a short-term decrease in leukocyte counts with β-chloroalkamine exposure, however there was no prolonged survival. Like the cytotoxic β-chloroalkamine found in mustard gas, busulfan is an alkylating

agent. In 1953, clinical trials were done to see the efficacy of busulfan versus radiotherapy and there was a somewhat longer survival in busulfan treated patients. Busulfan became the first approved chemotherapy (17).

Through the mid-20th century, the treatment for CML involved therapies that helped to keep the patient more comfortable. In the 1980s alpha-interferon (α-IFN) treatment was introduced and resulted in great clinical improvement. α-IFN therapy reduced the proliferation of leukemia cells, and even reduced the Philadelphia chromosome (discussed below) in some cases (18-20). The only curative therapy for CML is an allogeneic stem cell bone marrow transplant, however, the morbidity and mortality risks associated with this therapy make it a limited option (21, 22). Historical timeline of CML treatment is shown in Fig 1-2; current standards of care are discussed below.

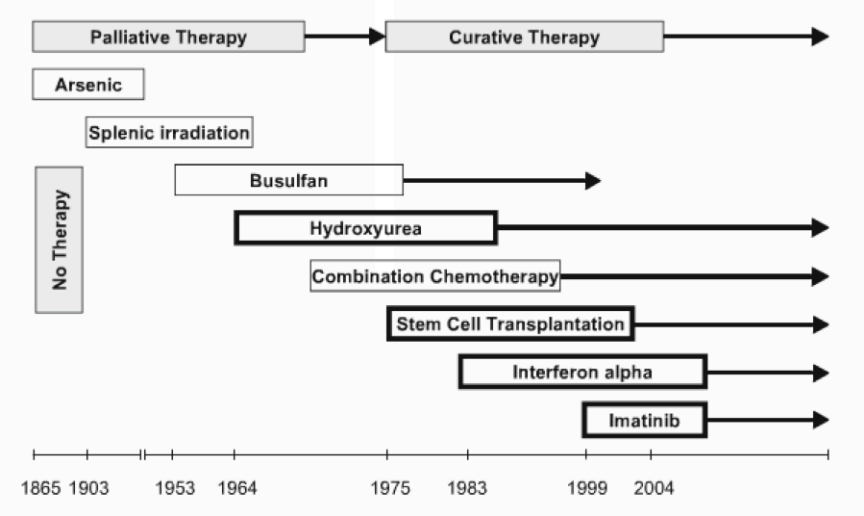

Figure **1-2**: Timeline of CML treatments.

Major treatments for CML over time are shown. Adapted from Hehlmann, *Annals of hematology*, 2005.

Philadelphia Chromosome

Discovery of BCR-ABL

Cancer is defined by the massive proliferation of non-functional cells, and the type of cancer is based on the tissue it develops, the rate of progression, and associated genetic mutations. BCR-ABL is not the only mutation that defines leukemia, nor is it the only defined genetic mutation associated with a specific cancer. The information discussed herein will only highlight BCR-ABL as a specific example of a genetic mutation and its association with CML.

In the 1960's in Philadelphia, Pennsylvania, two scientists changed the understanding of disease development. Drs. Peter Nowell and David Hungerford observed a consistent chromosomal abnormality in patients with CML. At the time, they made the bold statement that this abnormality was the causality of CML (23). However, it was not until 1973 that Dr. Janet Rowley identified the so-called Philadelphia (Ph) chromosome was from the reciprocal translocation between the long arms of chromosome 9 and 22 t(9:22)(q11) (24). This was the first time a chromosomal abnormality was associated with disease (Fig 1-3) (13, 25).

The causality of CML was highly debated, as viral infections were attributed to animal leukemias (8, 26). Independent laboratories showed the point of translocation in c-abl and limited breakpoints within a region, breakpoint cluster region (bcr), were distinct in humans, compared to other animals (26, 27). In 1990 it was confirmed that BCR-ABL was instrumental in CML (28). This was confirmed with the simultaneous generation of a mouse model to study the disease. Murine HSCs were transduced with retrovirus expressing BCR-ABL. Bone marrow transplantation of these cells caused a disease in lethally irradiated recipient mice, which was similar to that in humans (28).

Animal models of CML are used to study the disease *in vivo* (28, 29). Along with the retroviral model, one other common model is utilized. An inducible BCR-ABL model utilizes a double transgenic system in mice. One transgene expresses the tetracycline transactivator (tTA) protein from the Scl promoter, which is highly expressed in HSCs. The second transgene is the BCR-ABL oncoprotein under the control of the Tet responsive element (TRE). The binding of tTA to the TRE activates gene transcription. In the presence of tetracycline, or its analog doxycycline, the tTA has no activity. When doxycycline is removed, the tTA becomes active and BCR-ABL is expressed (30-32).

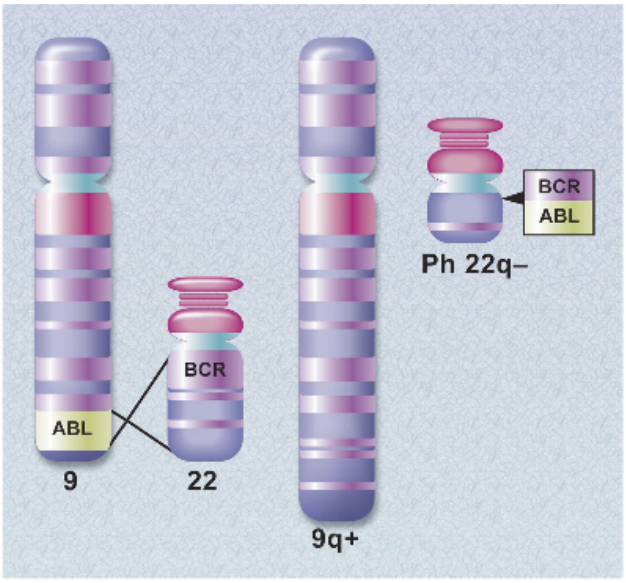

Figure **1-3**: The Philadelphia chromosome.

The ABL and BCR genes reside on the long arms of chromosome 9 and 22, respectively. As a result of the (9;22) translocation, a BCR-ABL gene is formed on the derivative chromosome 22. Figure from Druker, *Blood*, 2008. Illustration by A. Y. Chen.

Tyrosine kinase inhibitors

Tyrosine kinase inhibitors (TKIs) were the first rational therapy designed for a specific gene mutation (12). Though TKIs are not utilized in experiments discussed herein, their

development was based on the discovery that BCR-ABL kinase activity was essential in the progression of CML; specific inhibitors were designed for this target. TKIs were designed to inhibit the Abl kinase domain of BCR-ABL and are now widely considered the standards of care for CML (33, 34). In the mid 1990's, when these drugs were first being characterized, CGP 57148 (STI 571), a member of the 2-phenylaminopyrimidine class of tyrosine kinase inhibitors, emerged as the most potent inhibiter of Abl in intact cells (35, 36). Further studies showed STI 571 to suppress the proliferation of BCR-ABL expressing cells (37, 38). STI 571 was renamed and in 2001, imatinib mesylate (Gleevec) was the first FDA approved TKI therapy (37, 39, 40).

Until the 1980s, the treatment for CML included non-specific and highly toxic agents (41). α-IFN treatment was introduced in the 1970s and 1980s for treatment of both solid tumors and myeloid malignancies greatly improving outcome. The mechanism of action is complex and includes: induction of apoptosis, inhibition of cell growth, inhibition of angiogenesis, and suppression of hematopoiesis (42). It remained the standard of care for nearly two decades (19). However, the non-specific nature of the therapy resulted in off-target effects and a portion of CML patients was resistant to this therapy (43). Even today, this therapy is used in certain cases, in conjunction with TKIs (19).

Imatinib was introduced in 2001 for patients resistant or intolerant to α-IFN. It quickly became the front-line therapy to treat new cases of CML (44). Imatinib blocks ATP binding to the BCR-ABL tyrosine kinase and competitively inhibits the tyrosine kinase activity of BCR-ABL mediated signaling (45, 46), resulting in cell death (47). Inactivation of BCR-ABL-signaling significantly reduces myeloid cell proliferation in CML patients (46). Imatinib also inhibits other kinases including, c-kit, PDGFR, DDR1, and NQO2.

At the time of implementation, the thought was that imatinib could cure CML, as the BCR-ABL domain was the target. However, the cancer fights back against this therapy. Over time, many patients become resistant to treatment, through BCR-ABL gene mutation or

amplification (48). Though imatinib is still the first line of defense against newly diagnosed cases of CML, new therapies have been developed to counteract drug resistance.

Second and third-generation TKIs combat certain aspects of imatinib-resistance. They are effective against imatinib-induced BCR-ABL gene mutations, are higher potency, and have less off-target kinase inhibition. For example, the second generation TKI, dasatinib, can bind to both the active and inactive conformations of ABL kinase domain with 300 times the potency of imatinib (49-51). Ponatinib, a third-generation TKI, is the only TKI with activity against the highly drug-resistant T315I mutation (47).

Though TKIs have drastically increased the lifespan of CML patients, they are not a cure. Even in cases of CML where BCR-ABL is not mutated or amplified with TKI therapy, long-term treatment is required to suppress the disease. This is both costly and often manifests in off-target side effects; TKIs are highly specific, but also affect other kinases (46). As these therapies have only been in place for up to 16 years, long-term consequences of TKIs are unknown. There have been studies showing short-term discontinuation can result in relapse (52). The efficacy of TKI therapy centers on BCR-ABL signaling in the CML blast cells. Each generation of TKI is better in terms of specificity and potency (53). However, by design they will never eliminate the disease. A subset of CML cells, leukemia stem cells (LSCs), do not utilize BCR-ABL signaling for survival or proliferation and are resistant to TKI therapy. The milestones in CML biology, including the currently approved therapies, are highlighted in Table 1-1.

Table **1-1**: Milestones in unraveling the biology of CML

1842	Report of probably case of leukemia by Donne in Paris
1845	Recognition of leukemia (probably CML) as a disease entity
1846	First diagnosis of leukemia in a live patient
1880s	Development of methods for staining blood cells
1951	Dameshek introduces the concept of myeloproliferative disorders
1960	Identification of the Philadelphia chromosome (22q-)

1973	Recognition of the reciprocal nature of the (9;22) translocation
1984	Description of the breakpoint cluster region (bcr) on chromosome 22
1985	Identification of the BCR-ABL fusion gene and p210-Bcr-Abl
1989	Development of a reverse transcriptase PCR for measuring BCR-ABL transcripts
1990	Demonstration that the BCR-ABL gene can induce a CML-like disease in mice
1996	Demonstration of selective blocking of Bcr-Abl kinase activity
1998	Blocking Bcr-Abl kinase activity reverse features of CML
2001	Recognition of non-random mutations in the Abl kinase domain

Adapted from Goldman, 2010

Leukemia Stem Cells

Stem cells are defined as cells that have the ability to preserve themselves through self-renewal and to differentiate into more mature cells of a particular linage (54). HSCs are responsible for generation of blood forming and immune cells (55). Leukemias are the result of transformation of HSCs (56-58). The reciprocal translocation resulting in the BCR-ABL oncogene occurs in HSCs and gives rise to the LSC population. LSCs are similar to HSCs, but distinct in several ways, including their ability to "spontaneously" enter a state of continuous proliferation, both *in vitro* and *in vivo* to form blast cells, while a subset of LSCs self- renew and are maintained (59).

LSCs are generally defined clinically by the presence of surface antigens (60). These antigens play diverse functions in cellular classification and signaling, but in this context are discussed based on their presence only. In the peripheral blood of CML patients, LSCs are generally defined as lineage$^-$ (Lin$^-$) CD34$^+$CD38$^-$ leukocytes: CD34 is a marker antigen on HSCs and progenitors; CD38 is a maturation marker (60-63). Though controversial, cases of CD34$^-$ LSCs have been identified in AML (64). Other surface markers are used to characterize LSCs, though expression on CML LSCs is more variable, including: CD90 (Thy-1): a critical HSC

marker; CD33: myeloid lineage marker; CD117 (c-kit): receptor (tyrosine kinase) for stem cell factor; CD123 (IL3RA): often a strong marker for LSC (65); CLL-1: C-type lectin-like molecule-1, a stem cell marker (64). The variability of LSC surface markers indicates a heterogeneous population.

A defining characteristic of LSCs is the ability to give rise to disease in a new host (61). As with HSCs and progenitors, LSCs are sub-classified based on the balance of quiescence and differentiation. Sub-classes of suspected leukemia initiating cells can be transplanted into NOD/SCID mice to monitor disease (60, 66, 67). $CD34^{+}$ cells can be further divided by intracellular functional staining. High aldehyde dehydrogenase (ALDH) activity has been associated with leukemic long-term culture initiating cells (LTC-ICs) (60). Long-term LSCs are slowly dividing (PKH^{bright}), quiescent cells. Transplant of $CD34^{+}$, $ALDH^{bright}$, and/or PKH^{bright} cells correlates with cell survival and expansion in NOD/SCID mice (67).

In lethally irradiated wild-type mice, murine LSC-bone marrow transplantation leads to a well-defined model of CML (28). This allows the BCR-ABL oncogene to be functionally studied in order to develop and understand LSC-targeted therapies (33). While murine CML has a defined model of human disease, the HSC population (and LSC population) is different in terms of cell surface markers (66), and is mostly defined by the ability to generate disease in irradiated mice. In murine models, other markers, including sca-1^{+} and c-kit^{+} are used to define this population (68, 69). The LSC population in mice is thus defined as c-kit^{+}sca-$1^{+}Lin^{-}$ (KSL).

The LSC population is responsible for disease relapse in CML patients. TKIs target blasts, but do not affect the LSC population (62). In the non-proliferating state, LSCs do not relay on BCR-ABL signaling for survival or proliferation (70). Based on engraftment studies, it is clear that slow-dividing LSCs are the disease-causing cell population (66). These cells are highly resistant to apoptosis (71). In order to maintain the quiescent LSC phenotype, metabolic and cellular signaling is highly regulated. Current research is focused on targeting of LSC regulatory

pathways (59). The majority of LSC-targeted research has involved treatment with synthetic compounds that change the LSC phenotype (72). However, endogenous lipid mediators can also affect the implicated signaling pathways.

Diet and Leukemia

Dietary patterns are commonly studied for chemopreventive properties, though the relationship is complex (73, 74). One area of particular interest is dietary patterns in relation to generation of bioactive lipid mediators. Two dietary components, long-chain polyunsaturated fatty acids (PUFAs) and selenium, converge in the eicosanoid cascade to generate compounds with anti-leukemic functions.

Polyunsaturated fatty acids

PUFAs are essential macronutrients and include omega-3 and omega-6 fatty acids (75). The essential form of omega-3 is α-linolenic acid (ALA, C18:3; n-3) and omega-6 is linoleic acid (LA, C18:2; n-6). Major sources of ALA include walnuts, vegetable oils, and dark green leafy greens, with the adequate intake for adults being one to two grams per day. LA sources also include vegetable oils, nuts, seeds, animal products, and processed foods. LA is the most highly consumed PUFA in the Western diet, making up approximately six-percent of total energy (76, 77). The bioactive lipids of interest are 20 carbons in length: eicosapentaenoic acid (EPA, C20:5; n-3) and arachidonic acid (ARA, C20:4; n-6). In the case of ARA, the typical western diet has amble supply, as it is found in meat and eggs; EPA is found in fatty fish oils and algae. Another important PUFA in health and disease is docosahexaenoic acid (DHA, C22:6; n-3). Like EPA, it is abundant in fish and algae. The beneficial properties of DHA will only be addressed in the context of the omega-3 index.

Of particular importance in health is the ratio of omega-6:omega-3, with a lower ratio, i.e. higher omega-3 as beneficial (78). Unfortunately, the typical Western diet has increased this ratio over the late century (76). The elongation of an 18-carbon to 20-carbon fatty acid chain is not

highly efficient, but acts through the Δ6 elongation pathway (79), where additional carbons are added to the carboxyl end of the fatty acid. Each individual has unique requirements for nutrients. Genetic and environmental factors will affect the role of diet in health. Optimal health is the goal: knowledge based dietary interventions are beneficial in overall wellness.

In the body, PUFAs are incorporated into the phospholipid bilayer of cells and also stored in adipose tissue. The phospholipid bilayer is dynamic, but overall, the incorporation of esterified PUFAs is beneficial. In a clinical setting, the Omega-3 index quantifies EPA and DHA in the red blood cell (RBC) membrane. In humans, optimal Omega-3 index levels appear to be eight-percent or greater (80). Supplementation of EPA and/or DHA directly is necessary to achieve this level (81). The Omega-3 index is generally used to determine cardiovascular disease (CVD) risk: a low Omega-3 index is correlated with increased CVD risk. The Omega-3 index can also be studied in the context of cancer. Bioactive lipids have been indicated as both pro-and anti-cancer agents. In CML, a specific class of bioactive lipids changes the LSC phenotype.

Cyclopentenone prostaglandin biosynthesis

Eicosanoids are a unique class of bioactive lipids that are central in inflammatory responses. The properties of these compounds are cell-type specific. Eicosanoids are made of 20 carbons, and include prostaglandins (PGs), thromboxanes, leukotrienes, and epoxy derivatives. These important local mediators are implicated in diverse functions, including immune response, circadian rhythm, and gestation, to name a few (82-86). Though generally thought of as inflammatory, the function of PGs is complex. A unique class of PGs have an alkylidene cyclopentenone structure are called cyclopentenone prostaglandins (CyPGs). CyPGs are implicated in anti-proliferative functions (87).

Dietary PUFAs are incorporated in the phospholipid bilayer of immune cells. Upon stimulation, phospholipase A_2 (PLA_2) releases the free PUFA, for example ARA, into the intracellular space. Even though cyclooxygenases (COX) have no membrane-spanning transmembrane domains, they do associate with membranes (microsomal, mitochondrial, and perinuclear) through the atypical alpha-helices. COX-1 is thought to be constitutively expressed and acts to maintain homeostasis, while COX-2 is inducible upon inflammatory stimulation. COX-1/2 catalyzes the addition of two molecules of oxygen into ARA to form a highly reactive hydroperoxide, PGG_2. In addition to the cyclooxygenase active site, COX1/2 contain a heme group with peroxidase activity, which converts PGG_2 to PGH_2. PGH_2 is a common precursor that is utilized as a substrate by specific synthases, which catalyze the formation of a series of PGs (PGD_2, PGE_2, PGI_2, $PGF_{2\alpha}$) and thromboxane (TXA_2) (88) (Fig 1-4). Each of these metabolites has a unique, tissue specific role in modulation of inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-1/2. NSAIDs were developed to prevent the inflammatory mediators associated with COX activation. However, not all COX-derived lipids are bad. Not only are they self-regulating, but they also can act as anti-cancer agents (84, 89).

PGH_2 is converted to PGD_2 by two enzymes, hematopoietic PGD synthase (H-PGDS) and lipocalin-type PGD synthase (L-PGDS) that are predominantly expressed in the cells of the hematopoietic system and neuronal system, respectively. The dehydrated products of PGD_2, PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) belong to the class of cyclopentenone PGs (CyPGs). Members of this class, namely Δ^{12}-PGJ_2 and 15d-PGJ_2, have been shown to have anti-leukemic properties (87). Though other bioactive lipids are also important, only those derived following cyclooxygenase activity and prostaglandin synthases will be discussed herein.

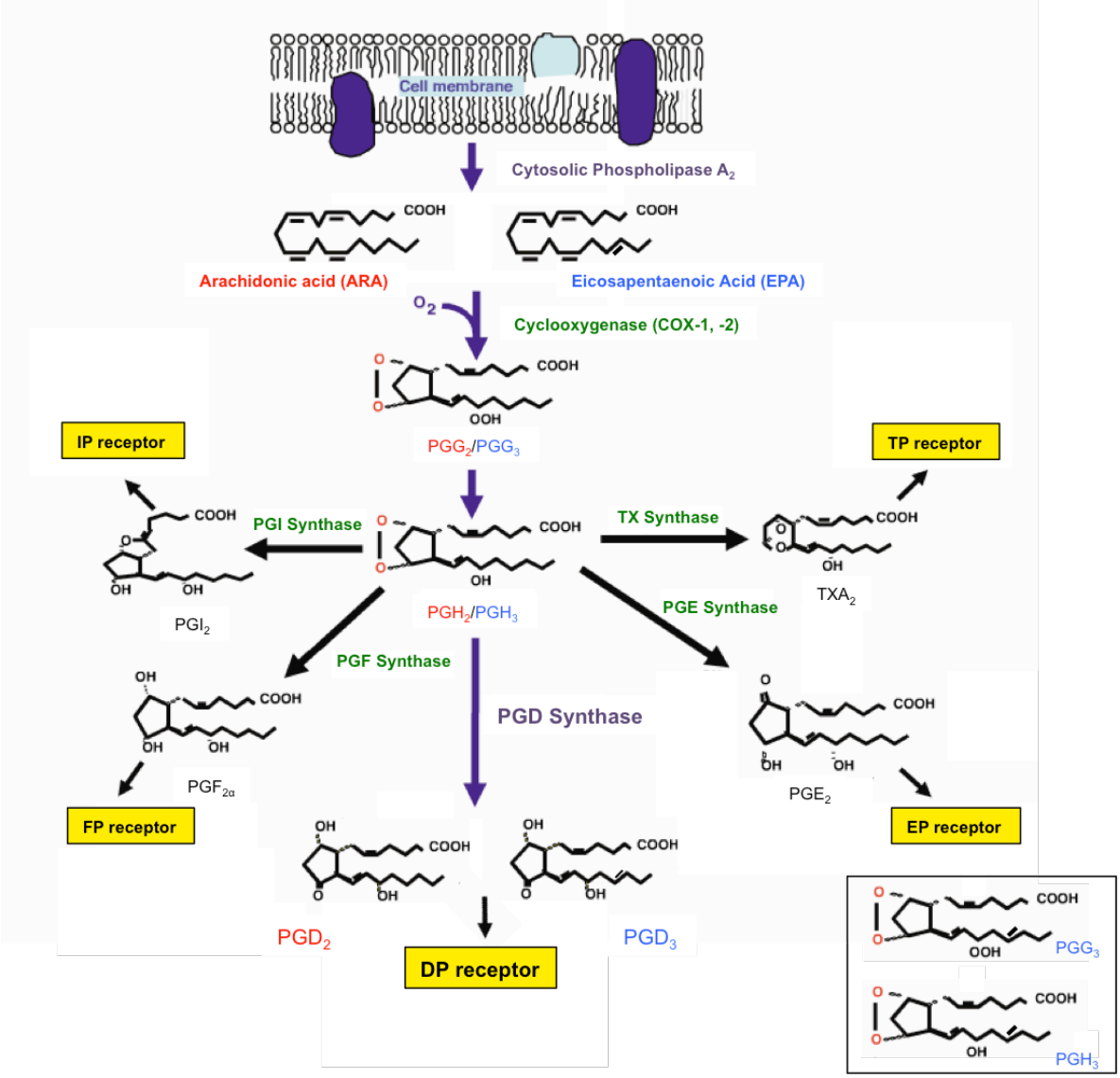

Figure **1-4**: Prostanoid biosynthesis schematic.

The key rate limiting steps are 1) liberation of arachidonic acid (ARA) or eicosapentaenoic acid (EPA) by the action of membrane bound phospholipase A_2 and 2) cyclooxygenase (COX) activity. In the case of EPA, only COX-2 can utilize the lipid for prostaglandin biosynthesis. Shown are the prostanoids and their respective membrane receptors. Inset shows structures of PGG_3 and PGH_3. Adapted from Dey et. al, *British Journal of Pharmacology*, 2006 (90).

Listed above is the conversion of ARA to PGs and TXA_2; EPA follows the same pattern. However, COX-1 cannot utilize EPA as a substrate, whereas COX-2 utilizes EPA with about 30% the efficiency of ARA (91). The difference in ARA and EPA is one additional double bond at the third carbon from the omega end in the latter, meaning the CyPGs derived from EPA have

an additional double bond. Eicosanoids derived from ARA are series-2; while EPA-derived are series-3 (Fig 1-5). Much like the CyPGs derived from ARA, CyPGs, Δ^{12}-PGJ_3 and 15d-PGJ_3 derived from EPA have shown efficacy in CML models (92).

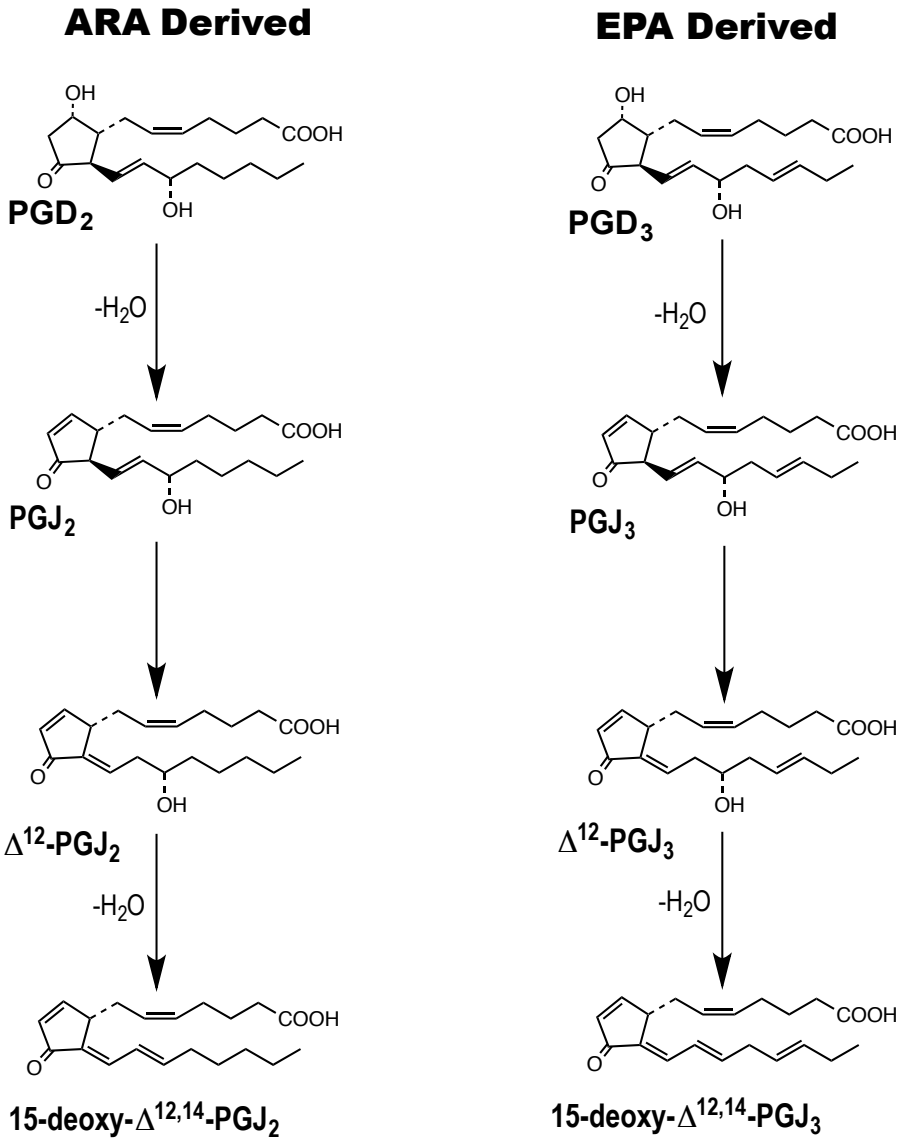

Figure **1-5**: J-family prostaglandins from arachidonic acid (ARA) and eicosapentaenoic acid (EPA).

Dehydration of D family prostaglandins into J family prostaglandins from ARA and EPA. 2-series PGs are derived from ARA; 3-series PGs from EPA. These reactions occur spontaneously downstream of prostaglandin synthase.

Despite the fact that EPA derived series 3 PGs bind to the same PG receptors (particularly PGE and TX receptors) as the series 2 from ARA, the former compounds demonstrate potent anti-inflammatory or pro-resolving properties as compared to their ARA-derived counterparts (84). Additionally, EPA incorporation is associated with reduced inflammation as it limits available ARA for COX. It is apparent that the ARA pathway is a double-edged sword and needs to be highly regulated. Based on the studies in our laboratory, the ability to skew ARA metabolism leading to the enhanced production of anti-inflammatory ARA-derived CyPGs while decreasing the levels of PGE_2 and TXA_2 is highly dependent on the levels of the micronutrient selenium, and the expression of selenoproteins, suggesting a redox control of ARA metabolism (68, 93, 94).

Selenium

Selenium is an essential micronutrient, generally found as its organic form, selenomethionine, in plants. When levels of selenium in the soil are adequate, good plant sources of selenium are Brazil nuts, seeds, leafy greens, and certain mushrooms (95). In areas where food distribution is well established, localized deficiencies of selenium in the soil can be mitigated. However, there are some geographical areas where the deficient soil leads to selenium related diseases.

Compared to the intake of many other micronutrients, the intake of selenium varies greatly worldwide. The US population has increased intake compared to Europe, a lot of this is due to over-taking supplements, many of which contain selenium (96). The estimated average requirement for selenium varies through the lifecycle. Individuals 19 years of age or older require

55 μg/day, with the tolerable upper intake (UTI) being 400 μg/day (97, 98). In our diets, 0.4 ppm equates to approximately 200 μg/day.

Certain areas of China, Tibet, Siberia, and North Korea have selenium deficient soil that has been associated with human health. Keshan disease is a congestive cardiomyopathy that occurs in combination with selenium deficiency and infection with a mutated Coxsackievirus. It was named after Keshan County of Heilongjiang province in Northeast China (99). The etiology of Kashin-Beck disease, characterized by inflammation in the cartilage, bone, and joints, is thought to be associated with selenium deficiency. In China, the intake of selenium is extremely variable, with some regions being deficient and others causing toxicity. Toxicity is associated with garlic like breath, hair and nail loss, disorders of the nervous system and skin, poor dental health, and paralysis (96). This indicates selenium as following a U-shaped curve in terms of its health benefits: deficient and high supplemented (> UTI) levels showing negative effects on health.

Selenoprotein synthesis:

Unlike other essential trace elements, selenium carries out its biological activity through a unique class of proteins: selenoproteins (100). Selenium, as selenocysteine (Sec), is the 21st amino acid. Sec has a structure almost identical to cysteine, except selenium in place of sulfur. Sec is incorporated into selenoproteins to affect anti-inflammatory and anti-oxidant functions. Twenty-five selenoproteins have been identified in humans and 24 in mice, with the function of several of these still unknown. The first report of Sec in a protein was in glutathione peroxidase 1 (GPX-1) (101). The synthesis of selenoproteins is highly regulated and unique.

Details of eukaryotic selenoprotein synthesis were modified from Bellinger et. al, 2009 (102), as follows. The tRNA for Sec ($tRNA^{Sec}$) recognizes the UGA codon, which normally acts as a stop codon in non-selenoproteins (100). Serine is conjugated to $tRNA^{Sec}$ by seryl-tRNA synthetase, and then modified to phosphoserine by phosphoseryl-tRNA kinase. Dietary selenium

is phosphorylated by selenophosphate synthetase 2 (SPS2) and then added to phosphoserine by selenocysteine synthetase to produce Sec. At the 3'-UTS of the mRNA, a SECIS element is required to recode the UGA stop codon for Sec insertion. The SECIS element is a unique stem-loop structure, which is a binding site for SBP2 (SECIS-binding protein 2). Biosynthesis of selenoproteins requires binding of SBP2 to the SECIS element and recruitment of the EFsec (Sec tRNA-specific elongation factor) bound to $tRNA^{Sec}$. The assembly of these factors on selenoproteins mRNAs in the nucleus sets the stage for decoding of UGA as Sec before export through the nuclear pore. Additional cofactors may contribute to selenoprotein synthesis (103, 104) (Fig 1-6).

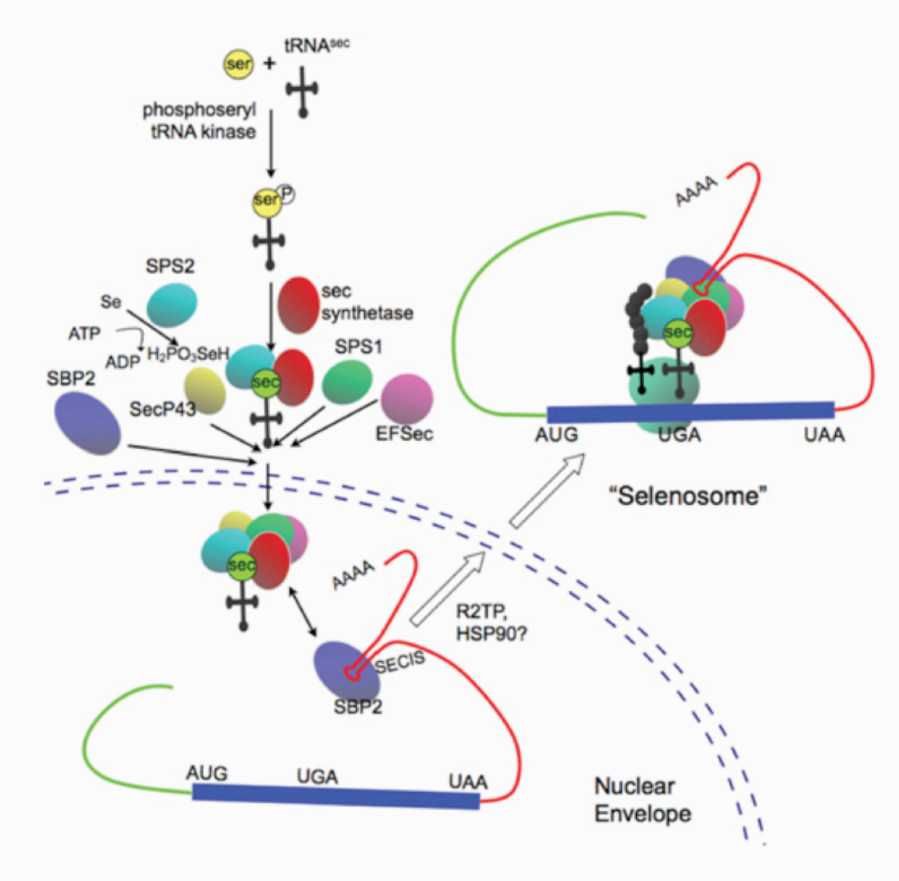

Figure **1-6**: Selenoprotein biosynthesis.

Biosynthesis of selenoproteins. SBP2, eEFSec, SLA, and SecP43 bind to the Sec insertion sequence (SECIS) element located in the 3' UTR of selenoproteins, forming a complex. This complex binds to the UGA codon, inducing translation and the production of selenoproteins (Adapted from Bellinger et. al, *Biochemical Journal*, 2009) (102).

Selenoproteins and leukemia

In the mid-20th century, a CML diagnosis meant measuring life in months, instead of years. Before understanding of selenoproteins, a small study from Case Western University investigated how reducing L-cysteine, with oral "selenium-cysteine" supplementation, reduced immature leukocytes in both AML and CML patients (105). Treatment with "selenium-cysteine" was toxic to the gastrointestinal tract, inducing nausea, vomiting, and diarrhea. This limited its study as a chemopreventative or therapy. At the time, it was proposed that "selenium-cysteine" inhibited certain enzymes necessary for cell growth and function. However, the mechanism of action was unknown (105). We now know that selenocysteine is incorporated into proteins important for redox mechanisms.

Given the presence of Sec in the active site of selenoproteins, these proteins have the ability to reduce reactive oxygen species (ROS), in the form of H_2O_2 and lipid-derived hydroperoxides, thus performing antioxidant functions. In LSCs, maintaining a low ROS level, through controlled antioxidant activity, is pivotal in maintaining quiescence. Many regulatory pathways, independent of selenium, help to maintain quiescent LSCs. However, a particular selenoprotein system has been shown to be important in LSCs. The TrxR-Trx redox couple contains Trx, selenoprotein thioredoxin reductase (TrxR), and the cofactor NADPH. Inhibition of Trx activity, with a histone methyltransferase, killed $Lin^-CD34^+CD38^-$ LSCs by reactivating with thioredoxin-interacting protein (TXNIP), the endogenous inhibitor of Trx1 (106). High Trx expression has also been shown in other cancer stem cells (CSCs), including breast cancer (107)

and lung cancer (108). This indicates redox regulation as central in several cancer progenitor populations.

ROS production in LSCs

In the 1920s, Otto Warburg demonstrated that in cancer cells, glycolysis dominates, even in the presence of oxygen, leading to excess production of lactate (109). In quiescent LSCs, the levels of glycolysis are lower than in bulk cells, but are still the dominant energy source. Glycolysis gives rise to less ROS and prevents further damage to intracellular membranes. ROS are endogenous byproducts of aerobic metabolism (110), which act as second messengers in many regulatory pathways: through the modulation of redox-sensitive upstream phosphatases and kinases. In HSCs, ROS causes oxidation of biomolecules such nucleic acids, lipids, and proteins. As a result, excessive ROS can impact cellular senescence, apoptosis, or may affect all major steps of carcinogenesis (111). In order to maintain low cellular levels of ROS in LSCs, several regulatory mechanisms are in place, where selenoproteins occupy a key role.

In recent years, the targeting of specific pathways has been suggested for eliminating LSCs. Of particular interest is in targeting pathways that affect “stem-like” characteristics. These include: the ability of self-renewal, quiescence, and independence of BCR-ABL for survival. However, the first step in CyPG regulation of ROS is determined by what receptor it first binds in the cell. Next, receptor-mediated pathways that are regulated by endogenous CyPGs are discussed. Endogenous CyPGs act through binding to a membrane G-protein coupled receptor (GPCR) or intracellular (PPARγ) receptors.

Receptor binding of prostaglandins

Prostaglandins are autacoids: they act temporary in not only in the cell in which they are produced, but also in neighboring cells. Macrophages are a major producer of PGs. In the CML microenvironment, PGs can be produced by LSCs or in nearby macrophages or granulocytes. Membrane transporters export PGs from cells into the extracellular milieu (Fig 1-7).

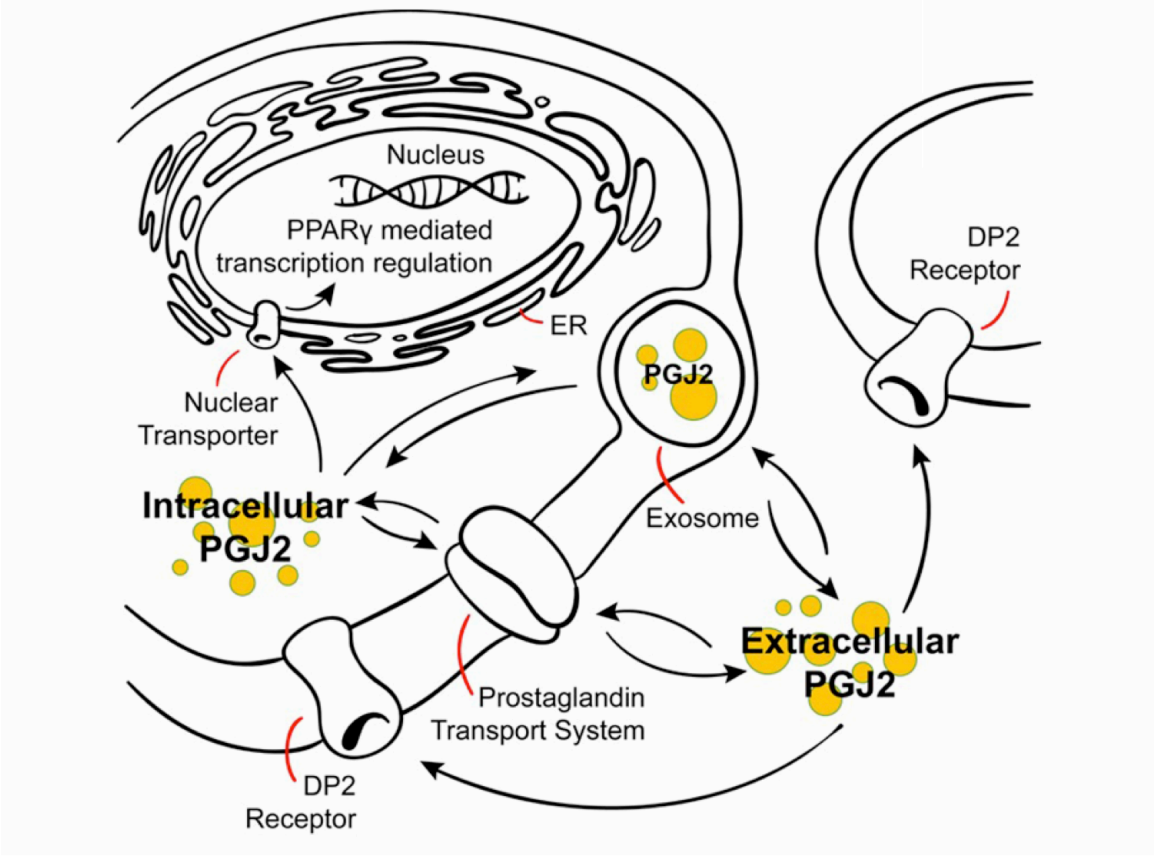

Figure **1-7**: J-family of prostanoids act as autacoids.

PGJ_2 (and PGJ_3) metabolites exit the cells via diffusion or transporters, and can enter cells or the nucleus via active transports at the plasma or nuclear membranes. PGs exert their actions by two main mechanisms: 1) binding to DP receptor (DP2/CRTH2 pictured) or 2) binding to peroxisome proliferator receptor (PPARγ) at the nuclear membrane. Trafficking of PGs in and out of cells can also occur via exosomes. Adapted from Figueiredo-Pereira et. al, 2015) (112).

DP Receptor

At the time of naming COX-derived bioactive lipids, it was hypothesized that each lipid had a unique and specific surface receptor (113). Parts of this were correct. As shown in Fig 1-4, each synthase enzyme, downstream of COX, gives rise to a unique class of lipids, binding to the related receptor. The key here is 'class' or type. Naming of membrane GPCRs followed that of prostanoid synthase enzymes. There is crossover binding, however the overall affinity is highest with the receptor originally named. The J-series CyPGs are dehydrated D-series products and bind with greatest affinity to D-series named receptors: DP1 (PGD_2 receptor) and $CRTH_2$ (chemoattractant receptor on Th2 cells), on effector cells.

DP1 is expressed in most tissues at low levels (114). This includes both immune cells and other tissues (epithelial goblet cells and smooth muscle cells). Activation of DP1 in granulocytes (eosinophils and mast cells) during allergies leads to degranulation. As a result, it also presents with bronchial constriction. Bronchial allergen challenge in asthmatic patients leads to rapid production of PGD_2 that can be detected within minutes in the bronchoalveolar lavage (BAL) fluid and is nearly 150 times higher than the pre-allergen levels (115). Though mostly implicated the role in allergies and asthma, the effect of DP1 receptor binding is diverse. For example, PGD_2 binding to DP1 in the brain regulates sleep (116); while in platelets it prevents aggregation (117).

CRTH2 was first identified on T-helper 2 (Th2) cells for their ability to recruit immune cells during an allergic response (118). This receptor is distinct from most prostanoid receptors; it is evolutionarily more similar to other chemoattractant receptors. It is expressed on inflammatory cells, including Th2 cells, eosinophils, and basophils, and induces chemotaxis (119). CRTH2 also plays an important role in cytokine release by Th2 cells, and degranulation of eosinophils (120). Intriguingly, it appears that some of these downstream functions that follow receptor activation are dependent on the ligand rather than simply receptor activation.

As summarized in Fig. 1-8, the DP1 and CRTH2 receptors signal downstream in cells via the intracellular messengers, cAMP and mobilization of Ca^{2+}, respectively. In humans, Δ^{12}-PGJ_2 can bind to both DP1 and CRTH2 with concentrations in the physiological range: Ki ~100 nM and ~7 nM for DP1 and CRTH2, respectively (121). However, the end effect of ligand binding to DP1 or CRTH2 is dictated by the type of ligand that binds (D-family, J-family; ARA- or EPA-derived) and the cellular environment. Interestingly, LSCs express both DP1 and $CRTH_2$ receptors.

Due to the central role of the DP1 and CRTH2 receptors in allergic diseases, including asthma, allergic rhinitis, and atopic dermatitis, synthetic antagonists have been developed to inhibit activation. One particular DP1 antagonist, BWA868C, has a Ki of 1.7 nM in humans (122). A CRTH2 antagonist, CAY10471, is highly selective, with a Ki of 0.6 nM in humans (123). As described in Chapter 4, these receptor antagonists will be extensively used to determine whether the endogenous anti-leukemic CyPGs act to alleviate leukemia via receptor-mediated binding. However, an alternative intracellular receptor, PPARγ, could also be responsible for the anti-leukemic functions of CyPGs.

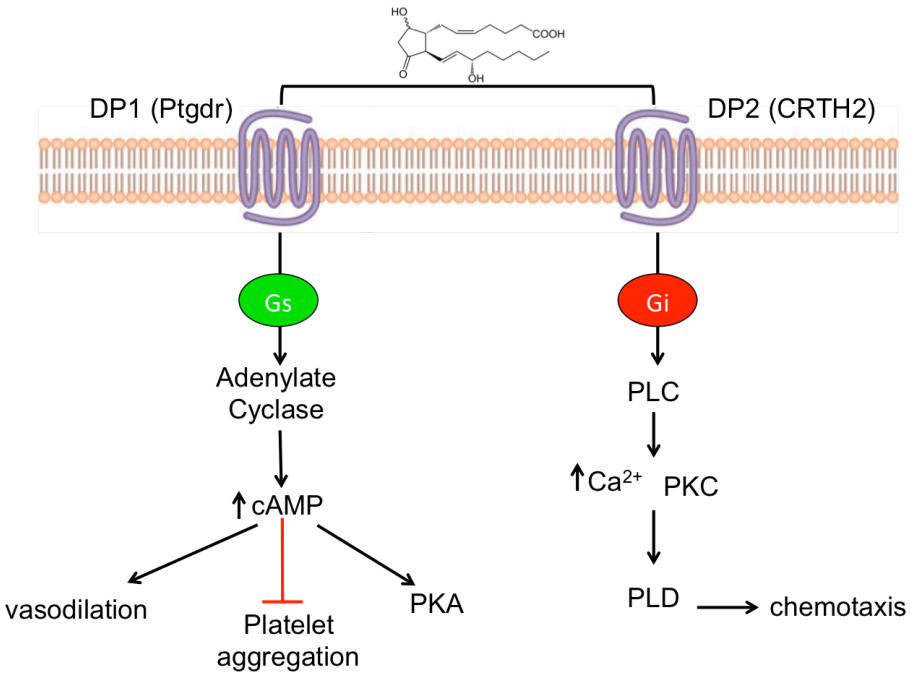

Figure **1-8**: DP1 and CRTH2 binding and immediate signaling by GPCR.

DP1 and CRTH2 mediated intracellular signaling. The affect of this signaling is cell type specific.

PPARγ

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone receptor superfamily. There are three subtypes: PPARα, PPARγ, and PPARδ/β, each of which has unique ligands, functions, and tissue distributions (124). Though originally thought to restrict to regulating glucose and lipid metabolism, PPARγ has recently been implicated in tumor growth (125). Of interest to us is the fact that PPARγ is suppressed in LSCs, but is expressed in further differentiated blast cells (126). Retinoid-X receptor (RXR) is a common heterodimeric protein that binds to PPARγ (127). The inactive form of PPARγ is found in the cytoplasm. Upon binding of the ligand to PPARγ, the heterodimer complex translocates to the nucleus to bind to the PPAR response element (PPRE) in the promoter of target genes (128). Such a liganded receptor displaces the NCoR co-repressor to recruit a co-activator such as histone

acetyltransferase p300, which along with RNA polymerase II form an active transcription complex.

PPARγ is able to bind numerous ligands. These include both synthetic drugs and endogenous metabolites. Thiazolidinediones (TZD), including pioglitazone, are synthetic drugs that are FDA approved for treatment of type 2 diabetes. CyPGs, 15d-PGJ_2 and Δ^{12}-PGJ_2, are also known agonists for PPARγ (83, 93, 129). It appears EPA-derived 15d-PGJ_3 and Δ^{12}-PGJ_3 can also activate PPARγ (92, 130).

PPARγ activation is involved in cellular proliferation, differentiation and apoptosis. *In vitro* studies showed a causal relationship between PPARγ and decrease in viable leukemia cells (131), with a focus on 15d-PGJ_2 and TZDs. Though informative, these studies were performed with immortalized leukemia cell lines with super- physiological levels of PPARγ ligands. However, recent *in vivo* studies revealed the potential translatability of these findings (132, 133). Activation of PPARγ in LSCs stimulates cells to exit quiescence. The mechanism involves manipulation of several gatekeepers, which regulate maintenance of the LSC population in the bone marrow niche (134). These include the signal transducer and activator of transcription-5a (STAT5a) and downstream transcription factors: CBP/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2) and hypoxia inducible factor 2α (HIF2α).

ROS-dependent signaling pathways in LSCs

STAT5

In BCR-ABL$^+$ CML and ALL, STAT5 is highly activated (135). The process of tumor maintenance is associated with rewiring of signaling pathways. Until recently, the role for STAT5 in CML has been implicated in the initiation phase *in vivo* (136). However, STAT5 appears to be important throughout the progression of disease. In BCR-ABL$^+$ leukemia, STAT5 is indispensable for maintenance, as STAT5 knockout mice effectively eliminated myeloid and lymphoid leukemia cells *in vivo* (137). The self-renewal property of HSCs is partially regulated by STAT5a phosphorylation (138). This property could extend to the quiescent state of LSCs. STAT5 levels have been shown to be abnormally high in LSCs (132). Additionally, STAT5 is a negative regulator of PPARγ (139). As PPARγ activation in LSCs may be an essential switch in turning quiescent LSCs to actively dividing cells, this implicates STAT5 as being important in this pathway.

ATM

Ataxia telangiectasia mutated (ATM) is a serine/threonine protein kinase, activated to aid in resolution of cellular damage. In the nucleus, ATM is activated by double-stranded DNA breaks (140); and in the cytoplasm by oxidative stress. The mechanism of action is dependent on the type of stress. In the nucleus, if damage cannot be repaired, ATM activates Chk2 and p53, leading to growth arrest or apoptosis (141). In the cytoplasm, ROS activation of ATM can lead to suppression of protein synthesis and increased autophagy. Defects in this pathway have been linked with cancers. Maintenance of ROS in LSCs is highly regulated. ATM signaling promotes production of NADPH though upregulation of the glucose-6-phosphate dehydrogenase. NAPDH helps to sequester excessive ROS (142). CyPGs appear to require the activity of ATM kinase to

effect the activation of p53 pathway in LSCs (73). This is most likely through increased ROS, due to loss of control of ROS-dependent transcription factors, including, HIF, NF-κB and p53.

ROS-dependent transcription factors in LSCs

HIF (hypoxia inducible factor)

Mammalian bone marrow (BM) is relatively hypoxic compared to other tissues. HIFs play a key role in HSC maintenance. HSCs concentrate in a hypoxic niche within the BM (143). Hypoxia is a well-characterized micro-environmental condition that helps to maintain LSCs, even in the presence of imatinib. The oxygen-sensitive HIF-α subunit binds to the constitutively expressed HIF-1β subunit and activates the transcription of target genes involved pathways such as glucose metabolism, erythropoiesis, and cell survival.

HIF-1α and HIF-2α are both inducible. Though literature is replete with the role of HIF-1α in leukemias, HIF-2α also has been suggested to regulate and maintain the LSC population (132). HIF-2α knockdown in primary AML samples decreased engraftment in receipt mice (144). Knockdown of HIF-2α, and to a lesser extent HIF-1α, impeded the long-term repopulating capacity of human $CD34^+$ umbilical cord blood cells. Hematopoietic stem and progenitor cells with HIF-2α knockdown showed increased ROS production and endoplasmic reticulum stress that triggered apoptosis through activation of the unfolded-protein-response (145). HIF-2α upregulates glycolysis in HSCs (138). HIF-1α may reprogram CSCs, by increasing anaerobic glycolysis, thus reducing ROS levels and increasing survival (143).

CITED2

CITED2 is essential for maintaining adult HSCs (146) and its deletion leads to rapid apoptosis (147). CITED2 is also important in the homing of HSCs and LSCs in the bone marrow.

CML cells express CITED2, with increased expression in the blast crisis stage (148). CITED2 competes with HIF-2α for CBP/p300 to enhance the expression of downstream target genes (147). In LSCs, CITED2 is regulated by STAT5. Interestingly, bioinformatics analysis shows CITED2 and NF-κB are located close together in the protein interaction network (149). This could mean the interaction in the nucleus of LSCs could be connected, but it is unclear how they regulate one another.

NF-κB

The redox-sensitive transcription factor NF-κB (nuclear factor kappa enhancer binding protein) is central to cellular survival, proliferation, immunity, and inflammation. Action of NF-κB leads to upregulation of survival signals in LSCs to prevent apoptosis. Inhibition of NF-κB in LSCs induces apoptosis (150). Of note, NF-κB is not detected in HSC and progenitors (151). This indicates NF-κB as being essential for the maintenance of LSCs, specifically. The suppression of oxidative stress by NF-κB in CSCs helps to maintain the population (152). Treatment with the sesquiterpene lactone, parthenolide, induced apoptosis in LSCs through inhibition NF-κB, excessive activation of ROS, and activation of p53 (72). Parthenolide preferentially targets LSCs over blast cells due to the high expression of NF-κB.

The subunits of NF-κB, p65 and p50, as a dimer, are maintained in the cytoplasm bound to the IκB inhibitory subunit. Phosphorylation by IκB kinase 2 (IKK2) leads to degradation of IκB by ubiquitination and degradation (153). This exposes the nuclear localization sequence in the p65-p50 heterodimer that ensures nuclear translocation for the binding to target gene promoters (154). PGD-derived CyPGs can inhibit NF-κB-dependent gene expression through two independent mechanisms. First, the cyclopentenone ring (carbon 9) in Δ^{12}-PGJ_2 and 15d-PGJ_2 (similar in PGD_3-derived CyPGs) act as a Michael acceptor to covalently modify the cysteine thiol group in the DNA binding domain of p65, in a process known as a Michael's addition reaction (155). Similarly, CyPGs inhibit the kinase activity of IKK2 by covalently binding to the

reactive ^{179}Cys-S^{-} in the active site of IKK2, thereby inhibiting the phosphorylation of IκB. This prevents NF-κB subunits from translocating into the nucleus. Second, ligand-dependent activation of PPARγ inhibits NF-κB -dependent expression of genes via squelching of p65 and p50 to keep them from binding to the NF-κB -binding site that leads to decreased expression of NF-κB target genes (156).

p53

p53 has been called a "cellular gatekeeper" (157) or "the guardian of the genome" (158), as it is central in coordinating the tumor-suppressor response to a broad range of cellular stress factors (159). p53 activity is important in cancer prevention. In many cancers, p53 mutations make it inactive or absent. p53 is regulated by multiple mechanisms: 1) p53 is repressed by the protein MDM2 (mouse double minute 2 homolog), which encodes a E3 ubiquitin-protein ligase that ubiquitinates the TAD (N-terminal transactivation domain) of p53 to clock its transcription activator function as well as aid in its degradation by proteasome; 2) Phosphorylation of p53, in the N-terminal domain, leads to its stabilization and activation. A broad range of kinases can modify p53, including ATM, ATR, Chk1, Chk2, and DNA-PK. Activation of p53 causes cell cycle arrest via the activation of p21 that negatively regulates the activity of cyclin-dependent kinases (CDKs).

Activation of either p53 or NF-κB due to cellular stress leads to very different outcomes. Where NF-κB initiates high glycolytic rates, p53 activates mitochondrial oxidative phosphorylation. In LSCs, p53 activation leads to apoptosis. This is likely through a complex cross talk between regulatory pathways. p53 inhibits NF-κB and switches from primary glycolytic energy production to oxidative phosphorylation, which exacerbates intracellular ROS production. This feeds-forward to further activate the ATM-p53 axis.

Research Hypothesis

A two-pronged approach, with diet and current TKI therapy, may be the key to elimination of all CML cells. In the context of diet in leukemia, bioactive lipids may be the key to reprogramming the quiescent LSC phenotype into a more active, and thus targetable, mature cell population. Pathways highlighted herein are not all those that have been investigated as targets in LSC-specific therapies. However, in the context of endogenous CyPGs, they appear most important. Here, we investigate the anti-leukemic role of bioactive lipids, which are produced *in vivo* with dietary manipulation. Dietary interventions in murine models of leukemia will be investigated, independently of TKI therapy. The idea is that if a dietary change alone decreases disease, this type of intervention, in conjunction with current TKI therapy, could mean a lesser duration and highly efficacious outcome of TKI therapy.

In this dissertation, the anti-leukemic activity of CyPGs, Δ^{12}-PGJ_2 and Δ^{12}-PGJ_3, will be investigated through binding to GPCRs, DP1/CRTH2 and/or PPARγ, as well as negatively affecting the activation of the redox-sensitive transcription factor, NF-κB. These studies are based on the overreaching central hypothesis that with ***endogenous CyPG production can be specifically enhanced through dietary manipulation and that the levels of endogenous CyPGs are able to eliminate the LSC population, resulting in a lesser disease burden.*** The hypothesis will be addressed in three *in vivo* models, two of CML and one of AML, which simulate human disease. Three major questions will be addressed:

1. Does supplementation with pharmacological doses of EPA increase endogenous Δ^{12}-PGJ_3 to levels to eliminate LSCs in experimental models of myeloid leukemias?
2. Is PPARγ activation required for the anti-leukemic effect of selenium supplementation, which is mediated through endogenous CyPGs?
3. Do CyPGs eliminate LSCs through binding to the DP1 and/or CRTH2 receptor?

References

1. Rai, K. R., and Jain, P. (2016) Chronic lymphocytic leukemia (CLL)-Then and now. *American journal of hematology* **91**, 330-340
2. Daley, G. Q. (2004) Chronic Myeloid Leukemia. *Cell* **119**, 314-316
3. Buechele, C., Breese, E. H., Schneidawind, D., Lin, C.-H. H., Jeong, J., Duque-Afonso, J., Wong, S. H., Smith, K. S., Negrin, R. S., Porteus, M., and Cleary, M. L. (2015) MLL leukemia induction by genome editing of human CD34+ hematopoietic cells. *Blood* **126**, 1683-1694
4. Slany, R. K. (2016) The molecular mechanics of mixed lineage leukemia. *Oncogene* [Epub ahead of print]
5. Saultz, J. N., and Garzon, R. (2016) Acute Myeloid Leukemia: A Concise Review. *Journal of clinical medicine* **5**, pii:E33
6. Geary, C. G. (2000) The story of chronic myeloid leukaemia. *British journal of haematology* **110**, 2-11
7. Bennett, J. H. (1852) Leucocythemia, or White cell blood, in relation to the physiology and pathology of the lymphatic glandular system. Sutherland and Knox, Edinburgh.
8. Goldman, J. M. (2010) Chronic myeloid leukemia: a historical perspective. *Seminars in hematology* **47**, 302-311
9. Wang, J. C., and Dick, J. E. (2005) Cancer stem cells: lessons from leukemia. *Trends in cell biology* **15**, 494-501
10. Ren, R. (2005) Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nature reviews. Cancer* **5**, 172-183
11. Calabretta, B., and Perrotti, D. (2004) The biology of CML blast crisis. *Blood* **103**, 4010-4022
12. Hehlmann, R., Hochhaus, A., and Baccarani, M. (2007) Chronic myeloid leukaemia. *The Lancet* **370**, 342-350
13. Druker, B. J. (2008) Translation of the Philadelphia chromosome into therapy for CML. *Blood* **112**, 4808-4817
14. Greaves, M. (2016) Leukaemia 'firsts' in cancer research and treatment. *Nature reviews. Cancer* **16**, 163-172
15. Forkner, C. E., and Scott, T. (1931) Arsenic as a therapeutic agent in chronic myelogenous leukemia: preliminary report. *JAMA* **97**, 3-5
16. O'Dwyer, M. E., La Rosée, P., Nimmanapalli, R., Bhalla, K. N., and Druker, B. J. (2002) Recent advances in Philadelphia chromosome-positive malignancies: the potential role of arsenic trioxide. *Seminars in hematology* **39**, 18-21
17. Silver, R. T., Woolf, S. H., Hehlmann, R., Appelbaum, F. R., Anderson, J., Bennett, C., Goldman, J. M., Guilhot, F., Kantarjian, H. M., Lichtin, A. E., Talpaz, M., and Tura, S. (1999) An evidence-based analysis of the effect of busulfan, hydroxyurea, interferon, and allogeneic bone marrow transplantation in treating the chronic phase of chronic myeloid leukemia: developed for the American Society of Hematology. *Blood* **94**, 1517-1536
18. Talpaz, M., McCredie, K. B., Mavligit, G. M., and Gutterman, J. U. (1983) Leukocyte interferon-induced myeloid cytoreduction in chronic myelogenous leukemia. *Blood*
19. Talpaz, M., Mercer, J., and Hehlmann, R. (2015) The interferon-alpha revival in CML. *Annals of hematology* **94 Suppl 2**, 207

20. Silver, R. T. (2006) Long-term effects of the treatment of polycythemia vera with recombinant interferon-alpha. *Cancer* **107**, 451-458
21. Goldman, J. M., Schmitz, N., Niethammer, D., and Gratwohl, A. (1998) Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe in 1998. Accreditation Sub-Committee of the European Group for Blood and Marrow Transplantation. *Bone marrow transplantation* **21**, 1-7
22. Goldman, J. (2008) Allogeneic stem cell transplantation for chronic myeloid leukemia-status in 2007. *Bone marrow transplantation* **42 Suppl 1**
23. Nowell, P. C., and Hungerford, D. A. (1960) Chromosome studies on normal and leukemic human leukocytes. *Journal of the National Cancer Institute* **25**, 85-109
24. Rowley, J. D. (1973) Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **243**, 290-293
25. Melo, J. V., and Barnes, D. J. (2007) Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nature reviews. Cancer* **7**, 441-453
26. Heisterkamp, N., Stephenson, J. R., Groffen, J., and Hansen, P. F. (1983) Localization of the c-abl oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. *Nature* **306**, 239-242
27. Groffen, J., Stephenson, J. R., Heisterkamp, N., and de Klein, A. (1984) Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* **36**, 93-99
28. Daley, G. Q., Etten, V. R. A., and Baltimore, D. (1990) Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* **247**, 824-830
29. Kelliher, M. A., McLaughlin, J., Witte, O. N., and Rosenberg, N. (1990) Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL. *PNAS* **87**, 6649-6653
30. Wertheim, J. A., Miller, J. P., Xu, L., He, Y., and Pear, W. S. (2002) The biology of chronic myelogenous leukemia:mouse models and cell adhesion. *Oncogene* **21**, 8612-8628
31. Huettner, C. S., Koschmieder, S., Iwasaki, H., Iwasaki-Arai, J., Radomska, H. S., Akashi, K., and Tenen, D. G. (2003) Inducible expression of BCR/ABL using human CD34 regulatory elements results in a megakaryocytic myeloproliferative syndrome. *Blood* **102**, 3363-3370
32. Koschmieder, S., and Schemionek, M. (2011) Mouse models as tools to understand and study BCR-ABL1 diseases. *American journal of blood research* **1**, 65-75
33. Hehlmann, R., Berger, U., and Hochhaus, A. (2005) Chronic myeloid leukemia: a model for oncology. *Annals of hematology* **84**, 487-497
34. Krause, D. S., and Van Etten, R. A. (2005) Tyrosine kinases as targets for cancer therapy. *The New England journal of medicine* **353**, 172-187
35. Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Müller, M., Regenass, U., and Lydon, N. B. (1995) Selective inhibition of the platelet-derived growth factor signal transduction pathway by a protein-tyrosine kinase inhibitor of the 2-phenylaminopyrimidine class. *PNAS* **92**, 2558-2562
36. Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Müller, M., Druker, B. J., and Lydon, N. B. (1996) Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer research* **56**, 100-104

37. Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J., and Lydon, N. B. (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr–Abl positive cells. *Nature Medicine* **2**, 561-566
38. Druker, B. J., and Lydon, N. B. (2000) Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *The Journal of clinical investigation* **105**, 3-7
39. Cilloni, D., and Saglio, G. (2012) Molecular pathways: BCR-ABL. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 930-937
40. Druker, B. J., Guilhot, F., O'Brien, S. G., Gathmann, I., Kantarjian, H., Gattermann, N., Deininger, M. W., Silver, R. T., Goldman, J. M., Stone, R. M., Cervantes, F., Hochhaus, A., Powell, B. L., Gabrilove, J. L., Rousselot, P., Reiffers, J., Cornelissen, J. J., Hughes, T., Agis, H., Fischer, T., Verhoef, G., Shepherd, J., Saglio, G., Gratwohl, A., Nielsen, J. L., Radich, J. P., Simonsson, B., Taylor, K., Baccarani, M., So, C., Letvak, L., Larson, R. A., and Investigators, I. (2006) Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *The New England journal of medicine* **355**, 2408-2417
41. Goldman, J. M., and Melo, J. V. (2003) Chronic myeloid leukemia--advances in biology and new approaches to treatment. *The New England journal of medicine* **349**, 1451-1464
42. Goldman, J. M., and Melo, J. V. (2001) Targeting the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *The New England journal of medicine* **344**, 1084-1086
43. Hochhaus, A., Lin, F., Reiter, A., Skladny, H., Mason, P. J., van Rhee, F., Shepherd, P. C., Allan, N. C., Hehlmann, R., Goldman, J. M., and Cross, N. C. (1996) Quantification of residual disease in chronic myelogenous leukemia patients on interferon-alpha therapy by competitive polymerase chain reaction. *Blood* **87**, 1549-1555
44. O'Brien, S. G., Guilhot, F., Larson, R. A., Gathmann, I., Baccarani, M., Cervantes, F., Cornelissen, J. J., Fischer, T., Hochhaus, A., Hughes, T., Lechner, K., Nielsen, J. L., Rousselot, P., Reiffers, J., Saglio, G., Shepherd, J., Simonsson, B., Gratwohl, A., Goldman, J. M., Kantarjian, H., Taylor, K., Verhoef, G., Bolton, A. E., Capdeville, R., Druker, B. J., and Investigators, I. (2003) Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *The New England journal of medicine* **348**, 994-1004
45. Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C. L. (2001) Efficacy and Safety of a Specific Inhibitor of the BCR-ABL Tyrosine Kinase in Chronic Myeloid Leukemia. *The New England Journal of Medicine* **344**, 1031-1037
46. Jain, P., Kantarjian, H., and Cortes, J. (2013) Chronic myeloid leukemia: overview of new agents and comparative analysis. *Current treatment options in oncology* **14**, 127-143
47. Apperley, J. F. (2015) Chronic myeloid leukaemia. *The Lancet*
48. Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N., and Sawyers, C. L. (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science (New York, N.Y.)* **293**, 876-880
49. Shah, N. P., Tran, C., Lee, F. Y., Chen, P., Norris, D., and Sawyers, C. L. (2004) Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science (New York, N.Y.)* **305**, 399-401
50. Copland, M., Hamilton, A., Elrick, L. J., Baird, J. W., Allan, E. K., Jordanides, N., Barow, M., Mountford, J. C., and Holyoake, T. L. (2006) Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. *Blood* **107**, 4532-4539

51. Jabbour, E., Jones, D., Kantarjian, H. M., O'Brien, S., Tam, C., Koller, C., Burger, J. A., Borthakur, G., Wierda, W. G., and Cortes, J. (2009) Long-term outcome of patients with chronic myeloid leukemia treated with second-generation tyrosine kinase inhibitors after imatinib failure is predicted by the in vitro sensitivity of BCR-ABL kinase domain mutations. *Blood* **114**, 2037-2043
52. Mahon, F.-X. X., Réa, D., Guilhot, J., Guilhot, F., Huguet, F., Nicolini, F., Legros, L., Charbonnier, A., Guerci, A., Varet, B., Etienne, G., Reiffers, J., Rousselot, P., and des Chroniques, I. (2010) Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *The Lancet. Oncology* **11**, 1029-1035
53. Kimura, S., Ando, T., and Kojima, K. (2014) Ever-advancing chronic myeloid leukemia treatment. *International journal of clinical oncology* **19**, 3-9
54. Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111
55. Arora, N., Wenzel, P. L., McKinney-Freeman, S. L., Ross, S. J., Kim, P. G., Chou, S. S., Yoshimoto, M., Yoder, M. C., and Daley, G. Q. (2014) Effect of developmental stage of HSC and recipient on transplant outcomes. *Developmental cell* **29**, 621-628
56. Fialkow, P. J., Gartler, S. M., and Yoshida, A. (1967) Clonal origin of chronic myelocytic leukemia in man. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 1468-1471
57. Fialkow, P. J., Jacobson, R. J., and Papayannopoulou, T. (1977) Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *The American journal of medicine* **63**, 125-130
58. Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M. A., and Dick, J. E. (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645-648
59. Crews, L. A., and Jamieson, C. H. (2013) Selective elimination of leukemia stem cells: hitting a moving target. *Cancer letters* **338**, 15-22
60. Buss, E. C., and Ho, A. D. (2011) Leukemia stem cells. *International journal of cancer. Journal international du cancer* **129**, 2328-2336
61. Dick, D. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Med* **3**, 730-737
62. Graham, S. M. (2002) Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* **99**, 319-325
63. Diaz-Blanco, E., Bruns, I., Neumann, F., Fischer, J. C., Graef, T., Rosskopf, M., Brors, B., Pechtel, S., Bork, S., Koch, A., Baer, A., Rohr, U. P. P., Kobbe, G., von Haeseler, A., Gattermann, N., Haas, R., and Kronenwett, R. (2007) Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients with CML in chronic phase. *Leukemia* **21**, 494-504
64. Taussig, D. C., Vargaftig, J., Miraki-Moud, F., Griessinger, E., Sharrock, K., Luke, T., Lillington, D., Oakervee, H., Cavenagh, J., Agrawal, S. G., Lister, T. A., Gribben, J. G., and Bonnet, D. (2010) Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood* **115**, 1976-1984
65. Jin, L., Lee, E. M., Ramshaw, H. S., Busfield, S. J., Peoppl, A. G., Wilkinson, L., Guthridge, M. A., Thomas, D., Barry, E. F., Boyd, A., Gearing, D. P., Vairo, G., Lopez, A. F., Dick, J. E., and Lock, R. B. (2009) Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. *Cell stem cell* **5**, 31-42

66. Goyama, S., Wunderlich, M., and Mulloy, J. C. (2015) Xenograft models for normal and malignant stem cells. *Blood* **125**, 2630-2640
67. Schubert, M., Herbert, N., Taubert, I., Ran, D., Singh, R., Eckstein, V., Vitacolonna, M., Ho, A. D., and Zöller, M. (2011) Differential survival of AML subpopulations in NOD/SCID mice. *Experimental hematology* **39**, 250-2630000
68. Gandhi, U. H., Kaushal, N., Hegde, S., Finch, E. R., Kudva, A. K., Kennett, M. J., Jordan, C. T., Paulson, R. F., and Prabhu, K. S. (2014) Selenium suppresses leukemia through the action of endogenous eicosanoids. *Cancer research* **74**, 3890-3901
69. Finch, E. R., Kudva, A. K., Quickel, M. D., Goodfield, L. L., Kennett, M. J., Whelan, J., Paulson, R. F., and Prabhu, K. S. (2015) Chemopreventive Effects of Dietary Eicosapentaenoic Acid Supplementation in Experimental Myeloid Leukemia. *Cancer prevention research (Philadelphia, Pa.)* **8**, 989-999
70. Corbin, A. S., Agarwal, A., Loriaux, M., Cortes, J., Deininger, M. W., and Druker, B. J. (2011) Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *The Journal of clinical investigation* **121**, 396-409
71. Holtz, M. S., Forman, S. J., and Bhatia, R. (2005) Nonproliferating CML CD34+ progenitors are resistant to apoptosis induced by a wide range of proapoptotic stimuli. *Leukemia* **19**, 1034-1041
72. Guzman, M. L., Rossi, R. M., Karnischky, L., Li, X., Peterson, D. R., Howard, D. S., and Jordan, C. T. (2005) The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. *Blood* **105**, 4163-4169
73. Berquin, I. M., Edwards, I. J., and Chen, Y. Q. (2008) Multi-targeted therapy of cancer by omega-3 fatty acids. *Cancer Letters* **269**, 363-377
74. Potter, J. D. (2014) The failure of cancer chemoprevention. *Carcinogenesis* **35**, 974-982
75. Flock, M. R., Harris, W. S., and Kris-Etherton, P. M. (2013) Long-chain omega-3 fatty acids: time to establish a dietary reference intake. *Nutrition reviews* **71**, 692-707
76. Blasbalg, T. L., Hibbeln, J. R., Ramsden, C. E., Majchrzak, S. F., and Rawlings, R. R. (2011) Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *The American journal of clinical nutrition* **93**, 950-962
77. Whelan, J. (2008) The health implications of changing linoleic acid intakes. *Prostaglandins, leukotrienes, and essential fatty acids* **79**, 165-167
78. Abedi, E., and Sahari, M. A. (2014) Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. *Food science & nutrition* **2**, 443-463
79. DiNicolantonio, J. J., McCarty, M. F., Chatterjee, S., Lavie, C. J., and O'Keefe, J. H. (2014) A higher dietary ratio of long-chain omega-3 to total omega-6 fatty acids for prevention of COX-2-dependent adenocarcinomas. *Nutrition and cancer* **66**, 1279-1284
80. Harris, W. S. (2010) The omega-3 index: clinical utility for therapeutic intervention. *Current cardiology reports* **12**, 503-508
81. Block, R. C., Harris, W. S., Reid, K. J., Sands, S. A., and Spertus, J. A. (2008) EPA and DHA in blood cell membranes from acute coronary syndrome patients and controls. *Atherosclerosis* **197**, 821-828
82. Lands, W. E., Libelt, B., Morris, A., Kramer, N. C., Prewitt, T. E., Bowen, P., Schmeisser, D., Davidson, M. H., and Burns, J. H. (1992) Maintenance of lower proportions of (n - 6) eicosanoid precursors in phospholipids of human plasma in response to added dietary (n - 3) fatty acids. *Biochimica et biophysica acta* **1180**, 147-162
83. Gilroy, D. W. (2010) Eicosanoids and the endogenous control of acute inflammatory resolution. *The international journal of biochemistry & cell biology* **42**, 524-528

84. Dennis, E. A., and Norris, P. C. (2015) Eicosanoid storm in infection and inflammation. *Nature reviews. Immunology* **15**, 511-523
85. Hoggatt, J., and Pelus, L. M. (2010) Eicosanoid regulation of hematopoiesis and hematopoietic stem and progenitor trafficking. *Leukemia* **24**, 1993-2002
86. Hayaishi, O., and Urade, Y. (2002) Prostaglandin D2 in sleep-wake regulation: recent progress and perspectives. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* **8**, 12-15
87. Scher, J. U., and Pillinger, M. H. (2005) 15d-PGJ2: the anti-inflammatory prostaglandin? *Clinical immunology (Orlando, Fla.)* **114**, 100-109
88. Smith, W. L., Garavito, R. M., and DeWitt, D. L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *The Journal of biological chemistry* **271**, 33157-33160
89. Morris, T., Rajakariar, R., Stables, M., and Gilroy, D. W. (2006) Not all eicosanoids are bad. *Trends in pharmacological sciences* **27**, 609-611
90. Dey, I., Lejeune, M., and Chadee, K. (2006) Prostaglandin E2 receptor distribution and function in the gastrointestinal tract. *British journal of pharmacology* **149**, 611-623
91. Wada, M., DeLong, C. J., Hong, Y. H., Rieke, C. J., Song, I., Sidhu, R. S., Yuan, C., Warnock, M., Schmaier, A. H., Yokoyama, C., Smyth, E. M., Wilson, S. J., FitzGerald, G. A., Garavito, R. M., Sui, D. X. e. X., Regan, J. W., and Smith, W. L. (2007) Enzymes and receptors of prostaglandin pathways with arachidonic acid-derived versus eicosapentaenoic acid-derived substrates and products. *The Journal of biological chemistry* **282**, 22254-22266
92. Hegde, S., Kaushal, N., Ravindra, K. C., Chiaro, C., Hafer, K. T., Gandhi, U. H., Thompson, J. T., van den Heuvel, J. P., Kennett, M. J., Hankey, P., Paulson, R. F., and Prabhu, K. S. (2011) Δ12-prostaglandin J3, an omega-3 fatty acid-derived metabolite, selectively ablates leukemia stem cells in mice. *Blood* **118**, 6909-6919
93. Gandhi, U. H., Kaushal, N., Ravindra, K. C., Hegde, S., Nelson, S. M., Narayan, V., Vunta, H., Paulson, R. F., and Prabhu, K. S. (2011) Selenoprotein-dependent up-regulation of hematopoietic prostaglandin D2 synthase in macrophages is mediated through the activation of peroxisome proliferator-activated receptor (PPAR) gamma. *The Journal of biological chemistry* **286**, 27471-27482
94. Mattmiller, S. A., Carlson, B. A., and Sordillo, L. M. (2013) Regulation of inflammation by selenium and selenoproteins: impact on eicosanoid biosynthesis. *Journal of nutritional science* **2**
95. Fairweather-Tait, S. J., Collings, R., and Hurst, R. (2010) Selenium bioavailability: current knowledge and future research requirements. *The American journal of clinical nutrition* **91**
96. Rayman, M. P. (2012) Selenium and human health. *Lancet (London, England)* **379**, 1256-1268
97. Kipp, A. P., Strohm, D., Brigelius-Flohé, R., Schomburg, L., Bechthold, A., Leschik-Bonnet, E., Heseker, H., and German. (2015) Revised reference values for selenium intake. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS)* **32**, 195-199
98. Prabhu, K. S., and Lei, X. G. (2016) Selenium. *Advances in nutrition (Bethesda, Md.)* **7**, 415-417
99. Wrobel, J. K., Power, R., and Toborek, M. (2015) Biological activity of selenium: Revisited. *IUBMB life*

100. Small-Howard, A. L., and Berry, M. J. (2005) Unique features of selenocysteine incorporation function within the context of general eukaryotic translational processes. *Biochemical Society Transactions* **33**, 1493-1497
101. Forstrom, J. W., Zakowski, J. J., and Tappel, A. L. (1978) Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry* **17**, 2639-2644
102. Bellinger, F. P., Raman, A. V., Reeves, M. A., and Berry, M. J. (2009) Regulation and function of selenoproteins in human disease. *The Biochemical journal* **422**, 11-22
103. Papp, L. V., Lu, J., Holmgren, A., and Khanna, K. K. (2007) From selenium to selenoproteins: synthesis, identity, and their role in human health. *Antioxidants & redox signaling* **9**, 775-806
104. Squires, J. E., and Berry, M. J. (2008) Eukaryotic selenoprotein synthesis: mechanistic insight incorporating new factors and new functions for old factors. *IUBMB life* **60**, 232-235
105. Weisberger, A. S., and Suhrland, L. G. (1956) Studies on analogues of L-cysteine and L-cystine. III. The effect of selenium cystine on leukemia. *Blood* **11**, 19-30
106. Ding, S., Li, C., Cheng, N., Cui, X., Xu, X., and Zhou, G. (2015) Redox Regulation in Cancer Stem Cells. *Oxidative medicine and cellular longevity* **2015**, 750798
107. Kim, S. J. (2005) High Thioredoxin Expression Is Associated with Resistance to Docetaxel in Primary Breast Cancer. *Clinical Cancer Research* **11**, 8425-8430
108. Soini, Y., Kahlos, K., and Näpänkangas, U. (2001) Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin Cancer Res* **7**, 1750-1757
109. Warburg, O., Wind, F., and Negelein, E. (1927) The metabolism of tumors in the body. *The Journal of general physiology* **8**, 519-530
110. Kobayashi, C. I., and Suda, T. (2012) Regulation of reactive oxygen species in stem cells and cancer stem cells. *Journal of cellular physiology* **227**, 421-430
111. Balaban, R. S., Nemoto, S., and Finkel, T. (2005) Mitochondria, oxidants, and aging. *Cell* **120**, 483-495
112. Figueiredo-Pereira, M. E., Rockwell, P., Schmidt-Glenewinkel, T., and Serrano, P. (2014) Neuroinflammation and J2 prostaglandins: linking impairment of the ubiquitin-proteasome pathway and mitochondria to neurodegeneration. *Frontiers in molecular neuroscience* **7**, 104
113. Kennedy, I., Coleman, R. A., Humphrey, P. P., Levy, G. P., and Lumley, P. (1982) Studies on the characterisation of prostanoid receptors: a proposed classification. *Prostaglandins* **24**, 667-689
114. Boie, Y., Sawyer, N., Slipetz, D. M., Metters, K. M., and Abramovitz, M. (1995) Molecular Cloning and Characterization of the Human Prostanoid DP Receptor. *Journal of Biological Chemistry* **270**, 18910-18916
115. Murray, J. J., Tonnel, A. B., and Brash, A. R. (1986) Release of prostaglandin D2 into human airways during acute antigen challenge. *N Engl J Med* **315**, 800-804
116. Urade, Y., and Hayaishi, O. (1999) Prostaglandin D2 and sleep regulation. *Biochem Biophys Acta* **1436**, 606-615
117. Townley, R. G., and Agrawal, S. (2012) CRTH2 antagonists in the treatment of allergic responses involving TH2 cells, basophils, and eosinophils. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **109**, 365-374
118. Nagata, K., Tanaka, K., Ogawa, K., Kemmotsu, K., Imai, T., Yoshie, O., Abe, H., Tada, K., Nakamura, M., Sugamura, K., and Takano, S. (1999) Selective expression of a novel

surface molecule by human Th2 cells in vivo. *Journal of immunology (Baltimore, Md. : 1950)* **162**, 1278-1286
119. Ito, S., Terasaka, T., Zenkoh, T., Matsuda, H., Hayashida, H., Nagata, H., Imamura, Y., Kobayashi, M., Takeuchi, M., and Ohta, M. (2012) Discovery of novel and potent CRTH2 antagonists. *Bioorganic & medicinal chemistry letters* **22**, 1194-1197
120. Gervais, F. G., Cruz, R. P., Chateauneuf, A., Gale, S., Sawyer, N., Nantel, F., Metters, K. M., and O'Neill, G. P. (2001) Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP. *The Journal of allergy and clinical immunology* **108**, 982-988
121. Sawyer, N., Cauchon, E., and Chateauneuf, A. (2002) Molecular pharmacology of the human prostaglandin D2 receptor, CRTH2. *Br J Pharacol* **137**, 1163-1172
122. Giles, H., Leff, P., Bolofo, M. L., Kelly, M. G., and Robertson, A. D. (1989) The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *British journal of pharmacology* **96**, 291-300
123. Ulven, T., and Kostenis, E. (2005) Minor structural modifications convert the dual TP/CRTH2 antagonist ramatroban into a highly selective and potent CRTH2 antagonist. *Journal of medicinal chemistry* **48**, 897-900
124. Schoonjans, K., Martin, G., Staels, B., and Auwerx, J. (1997) Peroxisome proliferator-activated receptors, orphans with ligands and functions. *Current opinion in lipidology* **8**, 159-166
125. Paulitschke, V., Gruber, S., Hofstätter, E., Haudek-Prinz, V., Klepeisz, P., Schicher, N., Jonak, C., Petzelbauer, P., Pehamberger, H., Gerner, C., and Kunstfeld, R. (2012) Proteome analysis identified the PPARγ ligand 15d-PGJ2 as a novel drug inhibiting melanoma progression and interfering with tumor-stroma interaction. *PloS one* **7**
126. Yousefi, B., Samadi, N., Baradaran, B., Shafiei-Irannejad, V., and Zarghami, N. (2016) Peroxisome proliferator-activated receptor ligands and their role in chronic myeloid leukemia: Therapeutic strategies. *Chemical biology & drug design* **88**, 17-25
127. Michalik, L., Desvergne, B., and Wahli, W. (2004) Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nature reviews. Cancer* **4**, 61-70
128. Desvergne, B., and Wahli, W. (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocrine reviews* **20**, 649-688
129. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83**, 803-812
130. Lefils-Lacourtablaise, J., Socorro, M., Géloën, A., Daira, P., Debard, C., Loizon, E., Guichardant, M., Dominguez, Z., Vidal, H., Lagarde, M., and Bernoud-Hubac, N. (2013) The eicosapentaenoic acid metabolite 15-deoxy-δ(12,14)-prostaglandin J3 increases adiponectin secretion by adipocytes partly via a PPARγ-dependent mechanism. *PloS one* **8**
131. Wang, D., and DuBois, R. N. (2006) Prostaglandins and cancer. *Gut* **55**, 115-122
132. Prost, S., Relouzat, F., Spentchian, M., Ouzegdouh, Y., Saliba, J., Massonnet, G., Beressi, J.-P. P., Verhoeyen, E., Raggueneau, V., Maneglier, B., Castaigne, S., Chomienne, C., Chrétien, S., Rousselot, P., and Leboulch, P. (2015) Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature* **525**, 380-383
133. Apsel Winger, B., and Shah, N. P. (2015) PPARγ: Welcoming the New Kid on the CML Stem Cell Block. *Cancer cell* **28**, 409-411
134. Krause, D. S., Fulzele, K., Catic, A., Sun, C. C., Dombkowski, D., Hurley, M. P., Lezeau, S., Attar, E., Wu, J. Y., Lin, H. Y., Divieti-Pajevic, P., Hasserjian, R. P., Schipani, E.,

Van Etten, R. A., and Scadden, D. T. (2013) Differential regulation of myeloid leukemias by the bone marrow microenvironment. *Nature medicine* **19**, 1513-1517

135. Carlesso, N., Frank, D. A., and Griffin, J. D. (1996) Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *The Journal of experimental medicine* **183**, 811-820
136. Moriggl, R., Sexl, V., Kenner, L., Duntsch, C., Stangl, K., Gingras, S., Hoffmeyer, A., Bauer, A., Piekorz, R., Wang, D., Bunting, K. D., Wagner, E. F., Sonneck, K., Valent, P., Ihle, J. N., and Beug, H. (2005) Stat5 tetramer formation is associated with leukemogenesis. *Cancer cell* **7**, 87-99
137. Hoelbl, A., Schuster, C., Kovacic, B., Zhu, B., Wickre, M., Hoelzl, M. A., Fajmann, S., Grebien, F., Warsch, W., Stengl, G., Hennighausen, L., Poli, V., Beug, H., Moriggl, R., and Sexl, V. (2010) Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO molecular medicine* **2**, 98-110
138. Fatrai, S., Wierenga, A. T., Daenen, S. M., Vellenga, E., and Schuringa, J. J. (2011) Identification of HIF2alpha as an important STAT5 target gene in human hematopoietic stem cells. *Blood* **117**, 3320-3330
139. Prost, S., Le Dantec, M., Augé, S., Le Grand, R., Derdouch, S., Auregan, G., Déglon, N., Relouzat, F., Aubertin, A.-M. M., Maillere, B., Dusanter-Fourt, I., and Kirszenbaum, M. (2008) Human and simian immunodeficiency viruses deregulate early hematopoiesis through a Nef/PPARgamma/STAT5 signaling pathway in macaques. *The Journal of clinical investigation* **118**, 1765-1775
140. McKinnon, P. J. (2004) ATM and ataxia telangiectasia. *EMBO reports* **5**, 772-776
141. Ray, P. D., Huang, B.-W. W., and Tsuji, Y. (2012) Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signalling* **24**, 981-990
142. Cosentino, C., Grieco, D., and Costanzo, V. (2011) ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *The EMBO journal* **30**, 546-555
143. Eliasson, P., and Jönsson, J.-I. I. (2010) The hematopoietic stem cell niche: low in oxygen but a nice place to be. *Journal of cellular physiology* **222**, 17-22
144. Gezer, D., Vukovic, M., Soga, T., Pollard, P. J., and Kranc, K. R. (2014) Concise review: genetic dissection of hypoxia signaling pathways in normal and leukemic stem cells. *Stem cells (Dayton, Ohio)* **32**, 1390-1397
145. Rouault-Pierre, K., Lopez-Onieva, L., Foster, K., Anjos-Afonso, F., Lamrissi-Garcia, I., Serrano-Sanchez, M., Mitter, R., Ivanovic, Z., de Verneuil, H., Gribben, J., Taussig, D., Rezvani, H. R., Mazurier, F., and Bonnet, D. (2013) HIF-2α protects human hematopoietic stem/progenitors and acute myeloid leukemic cells from apoptosis induced by endoplasmic reticulum stress. *Cell stem cell* **13**, 549-563
146. Korthuis, P. M., Berger, G., Bakker, B., Rozenveld-Geugien, M., Jaques, J., de Haan, G., Schuringa, J. J., Vellenga, E., and Schepers, H. (2015) CITED2-mediated human hematopoietic stem cell maintenance is critical for acute myeloid leukemia. *Leukemia* **29**, 625-635
147. Kranc, K. R., Schepers, H., Rodrigues, N. P., Bamforth, S., Villadsen, E., Ferry, H., Bouriez-Jones, T., Sigvardsson, M., Bhattacharya, S., Jacobsen, S. E., and Enver, T. (2009) Cited2 is an essential regulator of adult hematopoietic stem cells. *Cell stem cell* **5**, 659-665
148. Radich, J. P., Dai, H., Mao, M., Oehler, V., Schelter, J., Druker, B., Sawyers, C., Shah, N., Stock, W., Willman, C. L., Friend, S., and Linsley, P. S. (2006) Gene expression changes associated with progression and response in chronic myeloid leukemia.

Proceedings of the National Academy of Sciences of the United States of America **103**, 2794-2799

149. Lou, X., Sun, S., Chen, W., Zhou, Y., Huang, Y., Liu, X., Shan, Y., and Wang, C. (2011) Negative Feedback Regulation of NF-κB Action by CITED2 in the Nucleus. *The Journal of Immunology* **186**, 539-548
150. Guzman, M. L., Neering, S. J., Upchurch, D., and Grimes, B. (2001) Nuclear factor-κB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* **98**, 2301-2307
151. Reinisch, A., Chan, S. M., Thomas, D., and Majeti, R. (2015) Biology and Clinical Relevance of Acute Myeloid Leukemia Stem Cells. *Seminars in hematology* **52**, 150-164
152. Meng, Q., Peng, Z., Chen, L., Si, J., Dong, Z., and Xia, Y. (2010) Nuclear Factor-κB modulates cellular glutathione and prevents oxidative stress in cancer cells. *Cancer letters* **299**, 45-53
153. Hayden, M. S., and Ghosh, S. (2008) Shared principles in NF-kappaB signaling. *Cell* **132**, 344-362
154. Chen, Z. J. (2005) Ubiquitin signalling in the NF-kappaB pathway. *Nature cell biology* **7**, 758-765
155. Surh, Y.-J. J., Na, H.-K. K., Park, J.-M. M., Lee, H.-N. N., Kim, W., Yoon, I.-S. S., and Kim, D.-D. D. (2011) 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2, an electrophilic lipid mediator of anti-inflammatory and pro-resolving signaling. *Biochemical pharmacology* **82**, 1335-1351
156. Pascual, G., and Glass, C. K. (2006) Nuclear receptors versus inflammation: mechanisms of transrepression. *Trends in endocrinology and metabolism: TEM* **17**, 321-327
157. Levine, A. J. (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331
158. Carson, D. A., and Lois, A. (1995) Cancer progression and p53. *The Lancet* **346**, 1009-1011
159. Kruse, J. P., and Gu, W. (2009) Modes of p53 regulation. *Cell* **137**, 609-622

Chapter 2

Chemopreventative effects of dietary eicosapentaenoic acid supplementation in experimental myeloid leukemias

Published in Cancer Prev Res

Cancer Prev Res October 2015 8:989-999; Published OnlineFirst August 19, 2015; doi:10.1158/1940-6207.CAPR-15-0050

Emily R. Finch[1,2], Avinash K. Kudva[2] , Michael D. Quickel[2], Laura L. Goodfield[1,2], Mary J. Kennett[2], Jay Whelan[3], Robert F. Paulson[2†], and K. Sandeep Prabhu[2†]

Affiliations:

1. Immunology and Infectious Diseases Graduate Program, The Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA.
2. Center for Molecular Immunology and Infectious Disease and Center for Molecular Toxicology and Carcinogenesis, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA.
3. Department of Nutrition, University of Tennessee, Knoxville, TN.

Contributions:

Emily R. Finch was the lead author and experimental scientist: experimental design and execution, all figures and data analysis, compiled manuscript. Avinash K. Kudva ran LC-MS/MS samples. Michael D. Quickel prepared retrovirus. Laura L. Goodfield aided in sample collection. Mary J. Kennett assessed and scored histopathology samples. Jay Whelan ran GS/MS samples and aided in diet formulation. Robert F. Paulson and K. Sandeep Prabhu advised on experimental design and manuscript review.

Abstract

Current therapies for treatment of myeloid leukemia do not eliminate leukemia stem cells (LSC), leading to disease relapse. In this study, we supplemented mice with eicosapentaenoic acid (EPA, C20:5), a polyunsaturated omega-3 fatty acid, at pharmacological levels, to examine if the endogenous metabolite, cyclopentenone prostaglandin delta-12 PGJ_3 (Δ^{12}-PGJ_3), was effective in targeting LSCs in experimental leukemia. EPA supplementation for eight weeks resulted in enhanced endogenous production of Δ^{12}-PGJ_3 that was blocked by indomethacin, a cyclooxygenase inhibitor. Using a murine model of chronic myelogenous leukemia (CML) induced by bone marrow transplantation of BCR-ABL-expressing hematopoietic stem cells, mice supplemented with EPA showed a decrease in the LSC population, reduced splenomegaly and leukocytosis, when compared to mice on an oleic acid diet. Supplementation of CML mice carrying the T315I mutation (in BCR-ABL) with EPA resulted in a similar effect. Indomethacin blocked the EPA effect and increased the severity of BCR-ABL-induced CML and decreased apoptosis. Δ^{12}-PGJ_3 rescued indomethacin-treated BCR-ABL mice and decreased LSCs. Inhibition of hematopoietic-prostaglandin D synthase (H-PGDS) by HQL-79 in EPA-supplemented CML mice also blocked the effect of EPA. In addition, EPA supplementation was effective in a murine model of acute myeloid leukemia. Supplemented mice exhibited a decrease in leukemia burden and a decrease in the LSC colony-forming unit (LSC-CFU). The decrease in LSCs was confirmed through serial transplantation assays in all disease models. The results support a chemopreventive role for EPA in myeloid leukemia, which is dependent on the ability to efficiently convert EPA to endogenous cyclooxygenase-derived prostanoids, including Δ^{12}-PGJ_3.

Introduction

Leukemia remains one of the leading causes of cancer death, despite established well-defined standards of care. Each year, an estimated 43,000 new cases of leukemia will be diagnosed and 22,000 people will die from the disease (1). This observation underscores the need for further research into alternative therapies. Previously, we have demonstrated that certain cyclopentenone prostaglandin (CyPG) metabolites of polyunsaturated fatty acids (PUFA) are effective in treating murine models of leukemia (2). While PUFAs comprised of omega-6 and omega-3 fatty acids are essential for healthy growth and development, the ratio of these lipids is of particular interest in disease (3). This is because prostanoids produced from eicosapentaenoic acid (EPA, C20:5; n-3) show increased anti-inflammatory or 'pro-resolving' functions when compared to some of their omega-6 derived counterparts (4-6). Furthermore, alternative pathways produce other bioactive anti-inflammatory lipid mediators, including resolvins and protectins, from EPA and docosahexaenoic acid (DHA, C22:6; n-3), respectively (7-9).

Membrane incorporation of dietary EPA provides the substrate for the production of CyPG, Δ^{12}-PGJ_3 that is of particular interest in our laboratory, given its anti-leukemic properties (2). Release of EPA from the membrane by phospholipase A_2 commits the PUFA to CyPG synthesis through COX activity; EPA is converted to PGH_3 (10). PGH_3 is acted upon by PG-synthases, such as PGD synthases hematopoietic (H-PGDS) and lipocalin type (L-PGDS), to produce PGD_3 that undergoes dehydration and isomerization to Δ^{12}-PGJ_3. However, in cells, arachidonic acid (ARA, C20:4; n-6) is the preferred substrate for COX-2, while EPA is utilized at approximately 30% efficiency of ARA by COX-2 (11). Thus to increase production of 3-series PGs, EPA must be saturated in the membrane by increasing intake of omega-3 fatty acids. It is therefore conceivable that inhibition of COX-2 by non-steroidal anti-inflammatory drugs (NSAIDs) or H-PGDS by HQL-79 can impact leukemia progression. However, the results of

epidemiological studies are inconclusive regarding the relationship between non-aspirin NSAID use and incidence of leukemia (12-14), necessitating further research before embarking on new studies.

Myeloid leukemias are myeloproliferative disorders that arise from mutations within the hematopoietic stem cell (HSC) population in the bone marrow (15). These mutations lead to defects in the generation of healthy myeloid-lineage cells. In chronic myelogenous leukemia (CML), the Philadelphia chromosome is the result of a 9:22 chromosomal translocation, which leads to a constitutively active tyrosine kinase fusion protein BCR-ABL that is found in over 90% of cases (15, 16). Current therapy for BCR-ABL^{+} CML includes treatment with tyrosine kinase inhibitors (TKI). However, this maintenance therapy has various negative side effects and targets bulk tumor cells along with healthy cells while leaving leukemia stem cells (LSCs) unharmed. Disease relapse occurs due to LSCs' ability to self-renew and differentiate into bulk tumor cells (16, 17). Additionally, many patients become resistant to the therapy (15, 18, 19). The T315I mutation in BCR-ABL confers resistance to nearly all TKI therapies, except ponatinib (20). Finding a new mechanism that is independent of ABL kinase inhibition is essential to eliminating the cancer cells. Acute myeloid leukemia (AML) is characterized by immature blood cells that grow quickly and progress to leukemia in a few months. However, AML is a more heterogeneous disease with multiple etiologies including translocations. One translocation involved the MLL locus and multiple partners that lead to pediatric AML. One common MLL translocation generates the MLL-AF9 fusion protein that can confer self-renewal ability on hematopoietic progenitor cells leading to aggressive leukemogenesis (21).

In the present study, we show that EPA supplementation of mice at pharmacological doses alleviates symptoms and signs of leukemia in models of CML, T315I CML, and MLL-AF9 AML. Our data further support the key role of COX/H-PGDS-pathway-derived endogenous

metabolite of EPA to specifically target the LSC population to correct leukocytosis, splenomegaly, and overall disease index in these models of experimental leukemia.

Materials and Methods

Animals

Three-week-old C57BL/6 mice (Taconic Biosciences, Hudson, NY) were randomly assigned to two dietary groups: animals were housed 3-4 mice per cage in a temperature- and humidity-controlled room with a 12-hour light-dark cycle. All mice were 11-16 weeks of age at sacrifice, unless noted otherwise. For most experiments total animal number was 8-12; this was the summation of 3-4 independent experiments run in at least biological triplicate. The Institutional Animal Use and Care Committee (IACUC) at The Pennsylvania State University preapproved all procedures.

Mouse diet

An energetically equivalent mouse diet, in terms of omega-3 fatty acid, equivalent the FDA approved omega-3 supplement Lovaza® was developed (22). Diets remained isocaloric to the base diet (AIN-76A; Research Diets, Inc., New Brunswick, NJ) with 5g of total fat (12% of total calories). A portion of the corn oil in the base diet was replaced with the ethyl esters of oleic acid (OA, C18:1 n-9) (100%) or EPA (>90%) (Nu-Chek Prep, Elysian, MN) at a level of 0.82% (w/w), or 1.8% of total energy (kcal) (Table S1). The OA diet was used as an experimental control diet. The diets were stored in individual portions in airtight Whirl-Pak® bags at -80°C until the day of feeding (to prevent oxidation). The mice were fed *ad libitum* with fresh diet daily. In all experiments, mice were sacrificed after overnight fast (12-16 hours).

Fatty acid analysis

Fatty acid methyl esters of the tissue phospholipids were prepared and analyzed as described previously (22), with the following modifications. Briefly, tissues were homogenized in ice-cold saline. Three ml of chloroform-methanol (1:2, v/v) were added to each homogenate, and lipids were extracted with chloroform (1ml) plus saline (1ml), followed by chloroform (1ml) (x2).

The internal standard 1,2 diheptadecanoyl-sn-glycero-3-phosphocholine (17:0) (Avanti Polar Lipids, Alabaster, AL) was added to each sample prior to lipid extraction. The pooled chloroform extracts were evaporated, resuspended in a small volume of chloroform, and subjected to thin layer chromatography (TLC). The phospholipids were separated by TLC on silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) with chloroform/methanol (8:1 v/v) as the solvent system (phospholipids remained at the origin). Bands corresponding to the phospholipids were scraped, transferred to screw-topped test tubes and saponified in the presence of 14% BF_3 in methanol (Thermo Scientific, Bellefonte, PA) at 86°C for 10 min, followed by the addition of 0.9% saline (2ml). Fatty acid methyl esters were isolated with equal volumes of hexane (x2), pooled and evaporated under nitrogen. The fatty acid methyl esters were resuspended in hexane and analyzed using a Hewlett Packard model 5890 series II gas chromatograph equipped with flame ionization detector and a DB23 fused silica capillary column (0.25mm x 30m) (Agilent Technologies, Santa Clara, CA) with hydrogen as the carrier gas, and temperature programming from 160°C to 250°C at 3.5°C/min. The fatty acid methyl esters were identified by comparing the retention times with those of known standards (NuChek Prep, Elysian, MN). The fatty acids are presented as mole percent.

EPA supplementation *ex vivo* to detect Δ^{12}-PGJ_3

Murine macrophage-like cell line RAW 264.7 (ATCC, Manassas, VA) was cultured as previously described (23). In brief, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 2mM L-glutamine, 100units/ml penicillin-G (Cellgro, Manassas, VA), and 100mg/ml streptomycin (Invitrogen, Carlsbad, CA). For routine culture, the cells were passaged every three days at a ratio of 1:10 as per the recommendations by ATCC. For experimental assays, 100mm plates were seeded with $2x10^6$ cells. Exogenous fatty acid treatment began when cells were approximately 40% confluent. EPA or OA as ethyl ester (Cayman Chemicals, Ann Arbor, MI) was incubated

with cell-culture grade and endotoxin-free bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in serum free DMEM for one hour at 37°C. Final concentration of EPA or OA in culture was 50mM conjugated to 1% (v/v) BSA. Cells were treated with fatty acid for at least three days, with media changed daily, prior to 50ng/ml lipopolysaccharide (LPS) stimulation for 12 hours. In cultures treated with 10mM indomethacin (Cayman Chemicals, Ann Arbor, MI), the NSAID was added at least two hours prior to addition of fatty acid (as EPA or OA conjugated to BSA). Indomethacin was added to culture daily and was present during LPS stimulation.

Protocol for harvesting and culturing bone-marrow-derived macrophages (BMDMs) was modified from previously described work from our laboratory (23, 24). In short, BMDMs were prepared by extracting femoral bone marrow plugs from C57BL/6 mice maintained on either EPA or OA supplemented diet (as described above) for eight weeks. These cells were cultured in the same medium as described earlier with the addition of 20%(v/v) L929 fibroblast-conditioned medium (as a source of macrophage colony-stimulating factor, M-CSF) for at least three days. No additional fatty acid was added when culturing BMDMs. On day three, LPS (50ng/ml) was added for 12 hours. Clarified media was collected for lipid extraction as described below.

LC-MS/MS-MRM analysis

The estimation of Δ^{12}-PGJ_3 was carried out as reported previously (25). Briefly, Δ^{12}-PGJ_3 was quantified using a HPLC system consisting of LC-20AD UFLC pumps with a SIL-20AC autosampler (Shimadzu Corporation, Columbia, MD), a Luna™ phenyl-hexyl analytical column (2×150mm, 3 μm) (Phenomenex, Torrance, CA) developed with a 30 min isocratic elution with methanol/ water (70:30 v/v) containing 0.1 % acetic acid at a flow rate of 150μL/min and injection volume of 50μL. The negative ion electrospray tandem mass spectrometric analysis was carried out using API 2000 triple quadruple mass spectrometer (AB Sciex, Foster City, CA) at unit resolution with multiple reaction-monitoring mode (MRM). The source temperature was maintained at 450°C, electrospray voltage was -4500V and the declustering potential was set at -

16V. Nitrogen was used as collision gas at -20eV and the dwell time was 150ms/ion. During MRM, Δ^{12}-PGJ_3 was measured by recording the signal for the transition of the deprotonated molecule of *m/z* 331 to the most abundant fragment ion with *m/z* 269. Data were acquired and analyzed using Analyst software program version 1.5 (AB Sciex, Foster City, CA).

Generation of experimental leukemias

To induce experimental CML and T315I CML, retroviral stocks were generated by transfecting HEK293T cells with either pMIG-BCR-ABL (a gift from Dr. Warren Pear, University of Pennsylvania, Philadelphia) or MIGR-BCR-ABL-315T/I (a gift from Dr. Ravi Bhatia, University of Alabama, Birmingham), respectively, with pEco for packaging using TransIT 293 reagent (Mirus Bio, Madison, WI). For experimental AML, pMIG-MLL-AF9 (a gift from Dr. Hong-Gang Wang, Penn State College of Medicine, Hershey) was used. Isolation and transduction of HSCs was performed as described earlier (2, 26). HSCs transduced with BCR-ABL virus are hereafter referred to as BCR-ABL LSCs (BCR-$ABL^+GFP^+Kit^+Sca1^+Lin^-$). T315I CML LSCs are similarly defined. AML LSCs have not been defined with regard to cell surface markers. For all AML experiments, total bone marrow was transduced and transplanted ($1x10^6$ total transduced cells) to establish the model. GFP (C57BL/6-Tg(UBC-GFP)30Scha/J) (27) bone marrow transplant into OA and EPA supplemented mice was done to determine whether EPA impacts overall engraftment.

BCR-ABL LSCs were isolated from spleen and bone marrow by FACS using the Influx Cell Sorter (BD Biosciences, San Jose, CA) as described earlier (2, 28). LSCs ($5x10^5$ cells) in 100ml of sterile phosphate buffered saline (PBS) were transplanted by retro-orbital injection into lethally irradiated (950 rads) recipient mice maintained on EPA or OA diet groups for at least eight weeks. Disease progression was monitored by complete blood count (CBC) analysis, evaluating peripheral blood for leukocytosis on a Hemavet 950FS equipped with a veterinary software program. All mice were sacrificed by day-14 post-transplant. Spleen and bone marrow

were isolated and used for characterization as described below. Any deviation from this transplantation system will be described in figure legends. In all experimental models, transduced bone marrow cells were passaged through mice at least two times prior to transplantation into OA or EPA supplemented mice.

Inhibition of Δ^{12}-PGJ_3 synthesis *in vivo*

Inhibition of COX-1/2 was accomplished by use of indomethacin (Cayman Chemicals, Ann Arbor, MI). Indomethacin (0.00325%, w/v) was dissolved in ethanol (0.5%, v/v) and administered by means of oral drinking water for one week prior to bone marrow transplantation with BCR-ABL LSCs, as described previously (23, 28, 29). In a subset of Δ^{12}-PGJ_3 rescue experiments, administration of exogenous Δ^{12}-PGJ_3 was performed as described previously (2). In brief, mice were given daily intraperitoneal (*i.p.*) injection of Δ^{12}-PGJ_3 (0.025mg/kg) in 500 ml of sterile PBS for seven days, starting at day-7 post-transplant until sacrifice at day-14. HQL-79 (Cayman Chemicals, Ann Arbor, MI) was used to inhibit the activity of H-PGDS. HQL-79 (30 mg/kg body weight) was administered by *i.p.* injection every other day (3-4 times/week) from day zero (day of transplant) until day 14 (total of seven injections). HQL-79 was resuspended in a solution of 0.05 mM citric acid and 11.1 mM hydroxypropyl beta-cyclodextrin (HPBCD; Sigma, St. Louis) and incubated for >10 minutes at 37 °C, until dissolved. Mice were injected with this formation within 10 minutes of preparation. All mice were euthanized on day 14 post-transplant.

Flow cytometry of CML LSCs

Whole spleen and bone marrow were collected and red blood cells (RBCs) were lysed with ACK lysis buffer (1.5mM NH_4Cl, 100mM $KHCO_3$, 10mM EDTA-2Na). Lineage negative cells were isolated following manufacturer's protocol (Stem Cell Technologies, Vancouver, British Columbia). Cells were stained for Ly-6A/E (sca-1) and CD117 (c-kit) (BD Biosciences, San Jose, CA). Lineage negative white blood cells (WBCs) and the presence of LSCs were analyzed on Accuri C6 or Fortessa LSR flow cytometers (BD Biosciences, San Jose, CA). KSL

(c-kit^{+}sca-1^{+}Lin^{-}) LSCs were defined as Lin^{-} selected cells that stained positive for c-kit, sca-1, and GFP (present in BCR-ABL plasmid).

LSC-Colony Forming Units (LSC-CFU)

Total splenocytes or bone marrow from leukemic mice were plated in M3231 Methocult (Stem Cell Technologies, Vancouver, British Columbia). Cell density is described in figure legends. Media was supplemented with IL-3 (2.5ng/ml; R&D Systems, Minneapolis, MN), stem cell factor (SCF; 50ng/ml; Gold Biotechnology, St. Louis, MO), growth differentiation factor 15 (GDF-15; 30ng/ml; Biomatik, Wilmington, DE), and sonic hedgehog (Shh; 25ng/ml; Gold Biotechnology, St. Louis, MO). On days 7-10, colonies were counted. LSC-colony forming units (LSC-CFU) were defined based on their size (large: >100 cells at high magnification), shape (perfectly circular), density (dark brown/black color), and edge (clearly defined edge with limited differentiation). Any variation from LSC-CFU formation was also identified: in some cases granulocyte-macrophage progenitor colony forming units (CFU-GM) were observed (30). Each biological sample was plated in triplicate. The average number of LSC colonies from each biological sample was tabulated for analysis.

Histopathology and TUNEL staining of tissue sections

Whole spleen tissue was fixed in 4% paraformaldehyde at time of sacrifice. Sections were prepared (5 micron thickness) and stained with hematoxylin and eosin (H&E) at the Animal Diagnostic Laboratory, Penn State University, University Park, PA. Experimental groups were blinded prior to being examined and scored by a board certified laboratory animal veterinarian with training in pathology. Six characteristics were evaluated: disruption of splenic architecture (DSA), tumor infiltrates (TI), mitotic figures (MF; scored as 0= rare to none, 1= 1-3 per high power field (hpf), 2= 3-6 per hpf, 3= 6-9 per hpf, 4= >7 per hpf), megakaryocytes (MK), extramedullary hematopoiesis (EMH), and necrosis. With the exception of MF, tissue characteristics were scored on a 0-4 scale as follows: 0= within normal limits, 1= minimally

increased/elevated/affected, 2= mildly increased/elevated/affected, 3= moderately increased/elevated/affected, 4= extensively or severely increased or affected.

TUNEL assay was subsequently performed on representative tissues following histopathological examination using the *In Situ* Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, IN). Tissue sections were deparaffinized in Histo-Clear™ (National Diagnostics, Atlanta, GA), washed in 100% ethanol, 95% ethanol, 70% ethanol, and distilled water. Slides were then boiled in 10mM sodium citrate pH6.0 with 0.05% Tween-20 and allowed to cool. TUNEL staining was performed according to the manufacturer's instructions. Images were collected using an Olympus BX51 fluorescence microscope (Olympus America, Center Valley, PA). Total fluorescent area ($TUNEL^+$ cells) of stained slides was determined using Image J software program (NIH, Bethesda, MD).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Unless noted, nonparametric tests, including one-way ANOVA and un-paired one-tailed student t-test, were used where appropriate. The OA group was used as the control, unless otherwise noted, for t-tests (comparing EPA only to OA only). For ANOVA, all groups were compared. Variation from these analyses is described in figure legends. Bars represent mean ± SEM: *$p<0.05$, **$p<0.01$, ***$p<0.001$, #$p<0.0001$. The Biostatistics, Epidemiology, and Research Design (BERD) Core of Penn State Clinical and Translational Sciences Institute (CTSI) provided statistical consulting.

Results

Effect of EPA diet and omega-3 index

Two experimental diets were designed such that the only difference between dietary groups was the replacement of a subset of the base diet (AIN76-A) corn oil with either EPA or OA as ethyl esters at 0.82% (w/w), or 1.8% of total kcal (Table 2-1). Mice on either diet consumed a similar amount of diet and gained weight at the same rate (Fig. 2-1A). A widely accepted method for assessing omega-3 status is the omega-3 index, which is the relative abundance of EPA + DHA in red blood cells (RBCs) (31). To examine if EPA supplementation led to changes in the membrane lipid composition in the spleen, liver, and RBCs, the omega-3 index was determined by GC-MS analysis (22, 31). The amount and type of omega-3 fatty acid esters increased with EPA supplementation, as shown by the membrane lipid composition of the spleen, liver and RBCs (Fig. 2-1B). Compared to OA control diet, EPA supplementation led to a significant increase in the omega-3 index in the membrane of all examined tissues (Fig. 2-1C and Table 2-2). The content of omega-3 in the liver amounted to 19.4% of the membrane with EPA supplementation versus 7.8% in OA supplemented mice, indicating a 2.5-fold increase. In the spleen, there was a 3.5-fold increase in the omega-3 in EPA mice; the omega-3 index was 11.1% in EPA mice of compared with 3.2% in OA mice. The largest increase was in the RBCs where EPA supplementation increased the omega-3 index to 5.5% versus 1.0%, a 5.5 fold increase. These data demonstrate that an EPA supplemented diet increases the omega-3 content of cells, which will allow us to determine whether these changes lead to changes in Δ^{12}-PGJ_3 production, which in turn will affect CML and AML progression.

Table **2-1**: Diet Composition

Product #	D10001		D05121504		D05121506	
	AIN-76A		**0.82% Oleic Acid**		**0.82% Eicosapentaenoic**	
	gram%	kcal%	gram%	kcal%	gram%	kcal%
Protein	20	21	20	21	20	21
Carbohydrate	66	68	66	68	66	68
Fat	5	12	5	12	5	12
Total		100		100		100
kcal/gram	3.9		3.9		3.9	
Corn Oil	50	450	41.8	376	41.8	376
Oleic Acid, ethyl ester	0	0	**8.2**	**74**	0	0
Eicosapentaenoic Acid, ethyl ester	0	0	0	0	**8.2**	**74**
Total	1000	3902	1000.05	3902	1000.05	3902

Diets were formulated to be energetically equivalent to AIN-76A (2) and were manufactured by Research Diets, Inc. (New Brunswick, NJ). Fatty acid as ethyl ester, OA control (100% pure) or EPA (>90% pure) replaced a subset of corn oil in experimental diets. Purity was determined by GC-MS/MS (Nucheck Prep, Elysian, MN).

Table **2-2**: Lipid composition of tissue in healthy EPA- or OA- supplemented mice

	OA			EPA			
	mean	SEM	n	mean	SEM	n	p-value
Liver							
16:0	23.57	1.12	10	26.44	0.22	10	0.001153
18:0	16.07	0.62	10	15.15	0.33	10	0.285524
18:1	13.73	1.15	10	10.28	0.31	10	0.0001
18:2 (n-6)	10.97	1.13	10	14.69	0.32	10	3.04E-05
20:4 (n-6)	24.45	1.21	10	12.12	0.46	10	2.6E-30
20:5 (n-3)	0.00	0.00	10	**4.09**	0.33	10	**5.02E-06**
22:5 (n-6)	3.17	0.33	10	0.00	0.00	10	0.00034
22:5 (n-3)	0.00	0.00	10	**1.83**	0.15	10	**0.036631**
22:6 (n-3)	7.87	0.43	10	**15.30**	0.27	10	**7.95E-15**
Spleen							
16:0	28.30	0.74	7	31.30	0.67	13	0.00023
18:0	16.06	0.37	7	13.54	0.47	13	0.001824
18:1	13.29	0.43	7	14.28	0.86	13	0.213152
18:2 (n-6)	7.00	0.12	7	8.67	0.35	13	0.037596
20:4 (n-6)	22.04	0.82	7	10.20	0.55	13	5.2E-31
20:5 (n-3)	0.00	0.00	7	**5.64**	0.25	13	**5.11E-11**
22:5 (n-6)	0.00	0.00	7	0.00	0.00	13	NA
22:5 (n-3)	3.12	0.17	7	**6.87**	0.50	13	**5.46E-06**
22:6 (n-3)	3.12	0.17	7	**5.44**	0.27	13	**0.004053**
RBC							
16:00	43.37	1.69	9	46.74	1.00	10	9.22E-05
18:00	13.69	0.38	9	12.68	0.24	10	0.226678
18:01	20.44	0.48	9	17.01	0.17	10	6.67E-05
18:2 (n-6)	8.13	0.40	9	9.50	0.41	10	0.101258
20:4 (n-6)	9.49	1.01	9	4.40	0.29	10	9.91E-09
20:5 (n-3)	0.00	0.00	9	**2.63**	0.17	10	**0.001986**
22:5 (n-6)	0.00	0.00	9	0.00	0.00	10	NA
22:5 (n-3)	0.00	0.00	9	**1.63**	0.10	10	**0.052565**
22:6 (n-3)	1.03	0.11	9	**2.91**	0.25	10	**0.02577**

Mice were maintained on diet for eight weeks prior to sacrifice. Data represent individual lipid profiling depicted in Fig. 1b (lipids are presented as mole %). Multiple unpaired t-tests were performed to compare amount of each fatty acid in the tissue. P-values were adjusted to account for multiple comparisons by Holm-Sidak method.

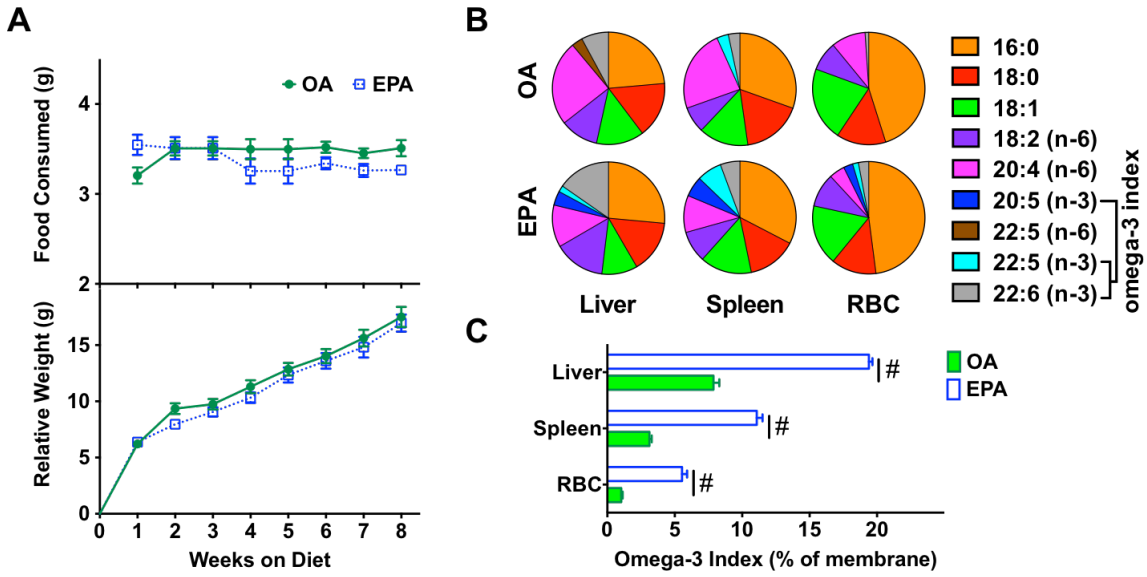

Figure **2-1**: EPA-supplemented diet increases the omega-3 index.

A, Food intake and relative body weight (compared to day 0) were monitored daily for eight weeks. n= 12 per diet group. **B**, Fatty acid composition of spleen, liver, and RBCs from OA- and EPA-supplemented mice by GC-MS. The fatty acids are presented as mole %. Data are represented as mean. **C**, Percent of membrane made of omega-3 fatty acids i.e. omega-3 index calculated from mean ± SEM. n= 7-12 per diet group. #p< 0.0001.

Endogenous Δ^{12}-PGJ$_3$ production

Based on our previously reported studies demonstrating the ability of Δ^{12}-PGJ$_3$ to specifically target LSCs (2), endogenous production of Δ^{12}-PGJ$_3$ was measured. EPA supplemented cells and mice were treated with LPS to mobilize membrane esterified EPA and to upregulate COX-2 activity (Fig. 2-2). As expected, EPA supplementation without LPS stimulation did not lead to detectable levels of Δ^{12}-PGJ$_3$ in either RAW 264.7 cells or BMDMs (data not shown). Primary BMDMs from mice on either OA or EPA supplemented diets were cultured *ex vivo* and stimulated with LPS. No additional fatty acid was added to the cultures. BMDMs derived from EPA supplemented mice produced significantly higher levels of Δ^{12}-PGJ$_3$

than OA derived BMDMs (Fig. 2-2A). We next tested whether Δ^{12}-PGJ_3 production was COX dependent. RAW 264.7 cells treated with EPA generated Δ^{12}-PGJ_3, in response to LPS. In contrast, cells treated with BSA (used as a carrier) alone or OA-conjugated BSA failed to produce any Δ^{12}-PGJ_3 (data not shown). As shown in Fig. 2-2B, indomethacin treatment blocked the production of Δ^{12}-PGJ_3 by EPA treated RAW 264.7 cells (Fig. 2-2B). Although EPA treated macrophages produced Δ^{12}-PGJ_3 *in vitro* in response to LPS treatment, we needed to determine whether mice fed on EPA supplemented diet produced significantly higher Δ^{12}-PGJ_3, *in vivo*, following LPS injection. Lipid extracts were isolated from plasma and subjected to LC-MS/MS analysis (Fig. 2-2C). While there was a non-significant increase in Δ^{12}-PGJ_3 in EPA mice versus OA mice without LPS treatment, stimulation with LPS for 12 hours led to a 3.5 fold increase in Δ^{12}-PGJ_3 in the plasma of EPA supplemented mice (Fig. 2-2C). Taken together, these results correlate an increase in the omega-3 index in EPA-fed mice with increased endogenous Δ^{12}-PGJ_3 production.

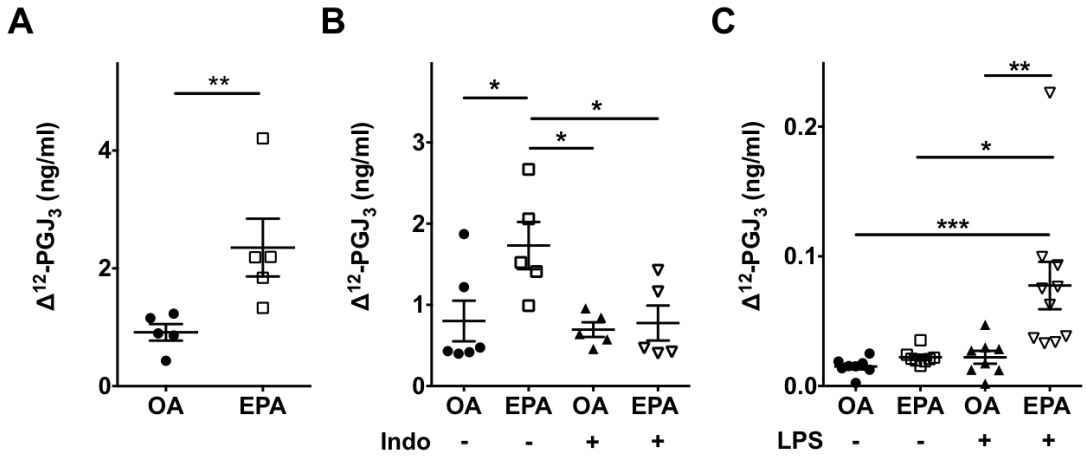

Figure **2-2**: Δ^{12}-PGJ_3 is produced in vivo and in vitro from exogenous EPA via the COX pathway.

A, Primary BMDMs were harvested from the femurs of EPA- or OA- supplemented mice (on diet for >8 weeks). Cells were cultured for four days and stimulated with 50ng/ml LPS for 12 hours prior to harvesting cells and media. Data represent one of five independent experiments. **B**, RAW 264.7 macrophages were treated for three days with 50mM fatty acid ethyl ester as OA or EPA- conjugated to BSA. Cells were treated with or without addition of 10mM indomethacin (Indo) for at least two hours prior to addition of fatty acid. LPS (50ng/ml) was added for 12 hours prior to collection of cells and media. Data represent two of five independent experiments. **C**, Plasma from healthy mice on supplemented diet was harvested after eight weeks on diet. LPS (1mg/kg body weight) was administered by i.p. injection 12 hours prior to harvesting blood. n= 8-10. Lipids were extracted from clarified media or whole plasma for LC-MS/MS analysis (MRM method) of Δ^{12}-PGJ_3. Individual data points are represented with error bars as the mean ± SEM. *p< 0.05, **p< 0.01, ***p< 0.001.

Supplementation with EPA affects CML

To relate increases in the omega-3 index and the ability of *in vivo* EPA supplementation to form endogenous Δ^{12}-PGJ_3 during CML, mice on the specific diets were given a bone marrow transplant of BCR-ABL^+ LSCs. As reported previously, transplantation with BCR-ABL LSCs leads to leukocytosis and splenomegaly (2, 28, 31). Interestingly, mice on an EPA supplemented diet showed less severe symptoms of BCR-ABL induced CML. Splenomegaly was decreased in EPA mice when compared to OA mice (Fig. 2-3A, Fig 2-5A), whereas healthy mice on diet showed no difference in spleen weight (Fig. 2-4A). Furthermore, H&E stained sections of whole spleen were scored based on six parameters of tissue health as mentioned in the "Materials and

Methods" section (Fig. 2-3B). OA supplemented LSC-transplanted mice had deterioration of normal spleen architecture with no clear demarcation between the red and white pulp areas (Fig. 2-3B, Fig. 2-5B). In contrast, H&E staining of splenic sections prepared from EPA supplemented LSC-transplanted mice showed normal structure in the spleen with well demarcated red and white pulp areas (Fig. 2-3B, Fig. 2-4B, Fig. 2-5B). To further demonstrate the effect of EPA in BCR-ABL induced CML, we examined the BCR-ABL LSC population in the spleen and bone marrow by flow cytometric analysis on day-14 post-transplantation. In both the spleen and bone marrow, OA-supplemented mice showed an approximate two-fold increase in the percentage of LSCs compared with EPA-supplemented mice (Fig. 2-3C). This increase in BCR-ABL LSCs was further corroborated with *ex vivo* investigation of BCR-ABL LSC-colony forming units (CFU) from the spleen and bone marrow. EPA mice failed to produce LSC-CFU colonies, whereas OA mice formed numerous LSC-CFUs (Fig. 2-3D). EPA mice exhibit dense colonies consistent with CFU-GM progenitor colonies (30). These assays showed that there were not only a limited number of LSCs in the EPA-supplemented group (as shown from flow cytometric results), but also these cells were not functional nor able to expand *ex vivo*.

A defining characteristic of LSCs is their ability to cause relapse of disease upon serial transplantation (32, 33). To further test the role of EPA in limiting experimental BCR-ABL induced CML, whole bone marrow from BCR-ABL LSC-primary-transplanted OA or EPA supplemented mice was used for a secondary transplant in mice fed on normal chow (Fig. 2-5C). The disease was allowed to progress for one month prior to analysis. This time point was chosen based on humane endpoints. At the time of sacrifice, recipients of leukemic OA bone marrow had lost 20% of body weight (Fig. 2-3E). Bone marrow from EPA supplemented BCR-ABL leukemic mice caused less severe disease as compared to OA supplemented mice, as shown by inhibition of leukocytosis (Fig. 2-3F, Fig 2-4C) and reduction in splenomegaly (Fig. 2-3G). Mice receiving OA leukemic bone marrow showed increased proliferation of splenocytes into LSC-CFUs (Fig.

2-3H). The number of colonies was too numerous to count (TNC) in all but one OA group, whereas the EPA group consistently showed limited cell growth (Fig. 2-3H). Taken together, these results supported our findings that EPA supplementation limited the LSC population in the spleen.

The T315I mutation in BCR-ABL is resistant to most current TKI therapeutics (20). These therapies are not only ineffective, but are also extremely damaging to healthy cells. EPA-supplemented mice transplanted with T315I bone marrow showed a decrease in disease, as compared to OA controls (Fig. 2-3I-J, Fig. 2-5D-F). Mice were monitored for overall survival and were euthanized based on humane endpoints, including drastic changes in behavior (data not shown) and in body weight (Fig. 2-5D). The experiment was ended at day 18 post-transplant. EPA mice showed a significant decrease in splenomegaly at time of sacrifice (Fig. 2-3I). Of particular interest, the EPA diet significantly limited the LSC population, as seen from LSC-CFU assay (Fig. 2-3J) and from flow cytometric analysis (Fig. 2-5F). No T315I LSC-CFUs formed *ex vivo* from EPA-supplemented bone marrow or spleen (Fig. 2-3J). These results suggest a potential breakthrough in limiting TKI-resistant CML with dietary change, where a COX-derived metabolite of EPA, such as Δ^{12}-PGJ_3, was likely mediating this process.

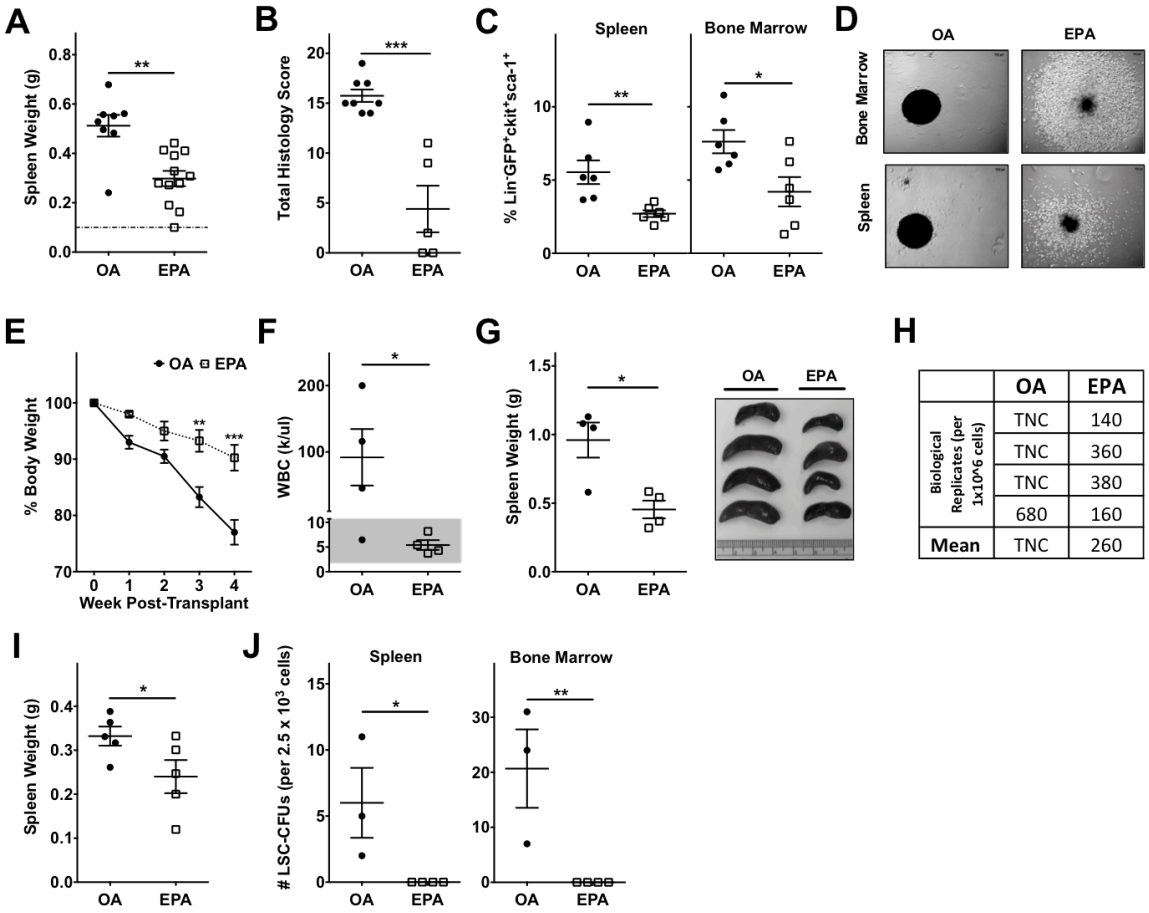

Figure **2-3**: EPA-supplemented mice show less severe signs and symptoms of experimental CML.

A-D: BCR-ABL$^+$ LSC transplanted mice; E-H: Secondary transplant model of total BCR-ABL bone marrow (See Fig. 2-5C for details); I-J: T315I transplant of 5x10^5 total T315I bone marrow. **A**, Spleen size in OA- and EPA-supplemented BCR-ABL$^+$ LSC transplanted mice. n= 8-12. Healthy spleen size: small dashed line (0.1g). **B**, Total histology scores of H&E stained spleen sections from OA- and EPA-supplemented BCR-ABL$^+$ LSC transplanted mice. Six parameters (DSA, TI, MF, MK, EMH, and necrosis) were scored from 0-4 and total scores are represented. n= 5-8 per diet group. **C,** Flow cytometric analysis of percentage of Lin$^-$ GFP$^+$c-kit$^+$sca-1$^+$ BCR-ABL LSCs in spleen and bone marrow. n= 6. **D,** Representative image of CML LSC-CFU assay from both spleen and bone marrow of OA- and EPA-supplemented BCR-ABL$^+$ LSC transplanted mice. Cells were plated at a concentration of 2x10^4 cells per mL. n= 3. **E,** Weight loss, presented as % body weight relative to week 0 post-secondary transplant of total BCR-ABL bone marrow. n= 4. **F**, Total WBC counts in peripheral blood (at 28 days post-secondary transplant of total BCR-ABL bone marrow). Shaded area (1.8-10.7 k/ml) indicates healthy range of WBC counts. n= 4. **G**, Spleen weight in secondary transplanted BCR-ABL mice. n= 4. **H**, Total BCR-ABL LSC-CFU counts (per 1x10^6 cells) in secondary transplanted BCR-ABL mice. Too numerous to count (TNC). n= 4. **I**, Spleen size in OA- and EPA-supplemented T315I total bone marrow transplanted mice. n= 5. **J**, Total T315I LSC-CFU counts in spleen and bone marrow from OA- and EPA-supplemented T315I transplanted mice. Cells were plated at the concentration of 2.5x10^3 cells per mL. n=4. Data points represent individual mice and bars represent mean ± SEM *p< 0.05, **p< 0.01, ***p< 0.001.

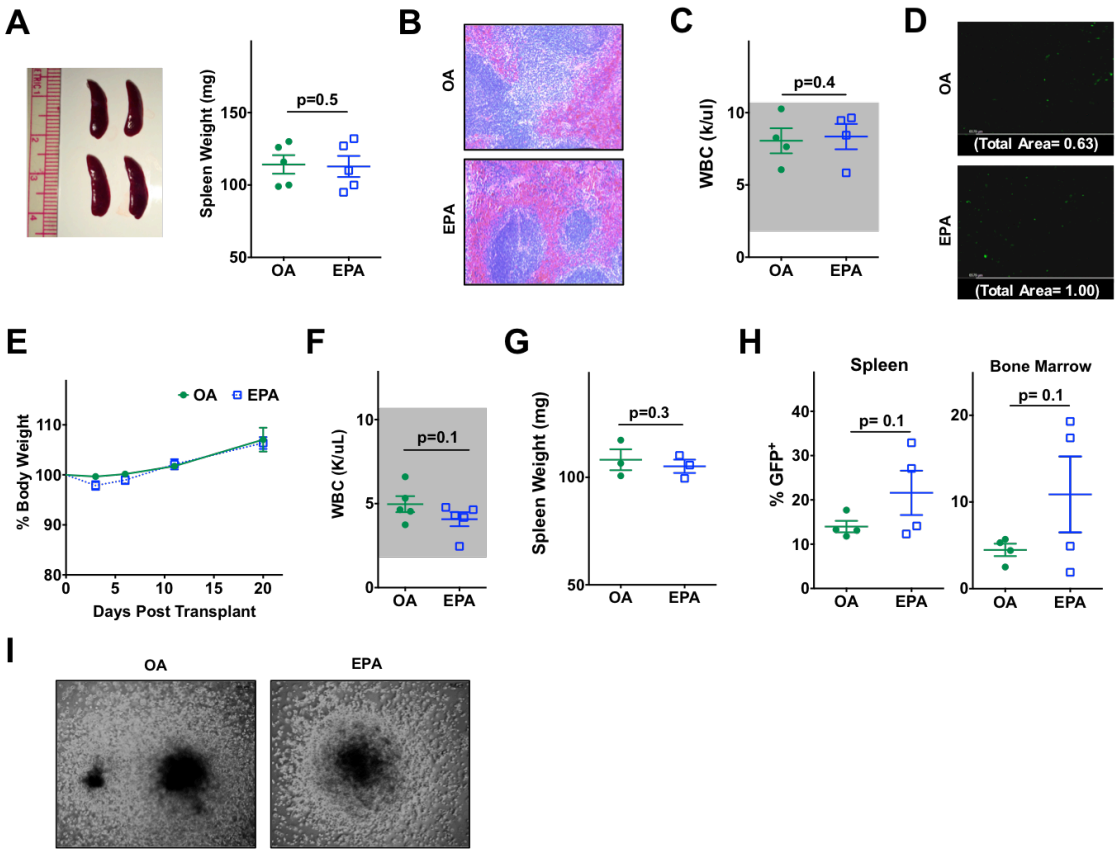

Figure **2-4**: Blood parameters in healthy mice are not affected by diet.

A-D: Healthy mice were maintained on EPA- or OA-supplemented diet for eight weeks prior to sacrifice; E-I: GFP transplanted mice. Irradiated mice were transplanted with $1x10^6$ total bone marrow cells from GFP mice (C57BL/6-Tg(UBC-GFP)30Scha/J) (1). **A**, Spleen weight in healthy EPA and OA supplemented mice. Data represent one of four independent experiments. n= 5. **B**, Example of H&E staining in EPA and OA supplemented mice. All six parameters (disruption of splenic architecture, tumor infiltrates, mitotic figures, megakaryocytes, extramedullary hematopoiesis and necrosis) were normal. **C**, Total WBC counts in healthy EPA- or OA-supplemented mice. Shaded area (1.8-10.7 k/ml) indicates healthy range of WBC counts. Data represent one of four independent experiments. n= 5. **D**, TUNEL assay of the splenic sections from healthy mice on EPA and OA supplemented diets. Representative images with TUNEL-positive staining are shown. Total area of $TUNEL^+$ staining is shown below each panel. n= 4. **E**, Body weight change in OA- and EPA- supplemented mice during healthy GFP^+ bone marrow transplant. n= 4. **F**, Peripheral blood WBC counts in OA- and EPA-supplemented mice on day 14 post-transplant with healthy GFP^+ bone marrow. n= 4. **G**, Spleen weight following GFP^+ bone marrow transplant in healthy OA- and EPA-supplemented mice. n= 4. **H**, Flow cytometric analysis of GFP engraftment in healthy OA and EPA supplemented mice in the bone marrow and spleen three weeks post-transplant. n= 4. **I**, Healthy CFU example from bone marrow of GFP transplanted mice. Representative image from n= 4 is shown. Data represent individual mice with error bars of mean ± SEM.

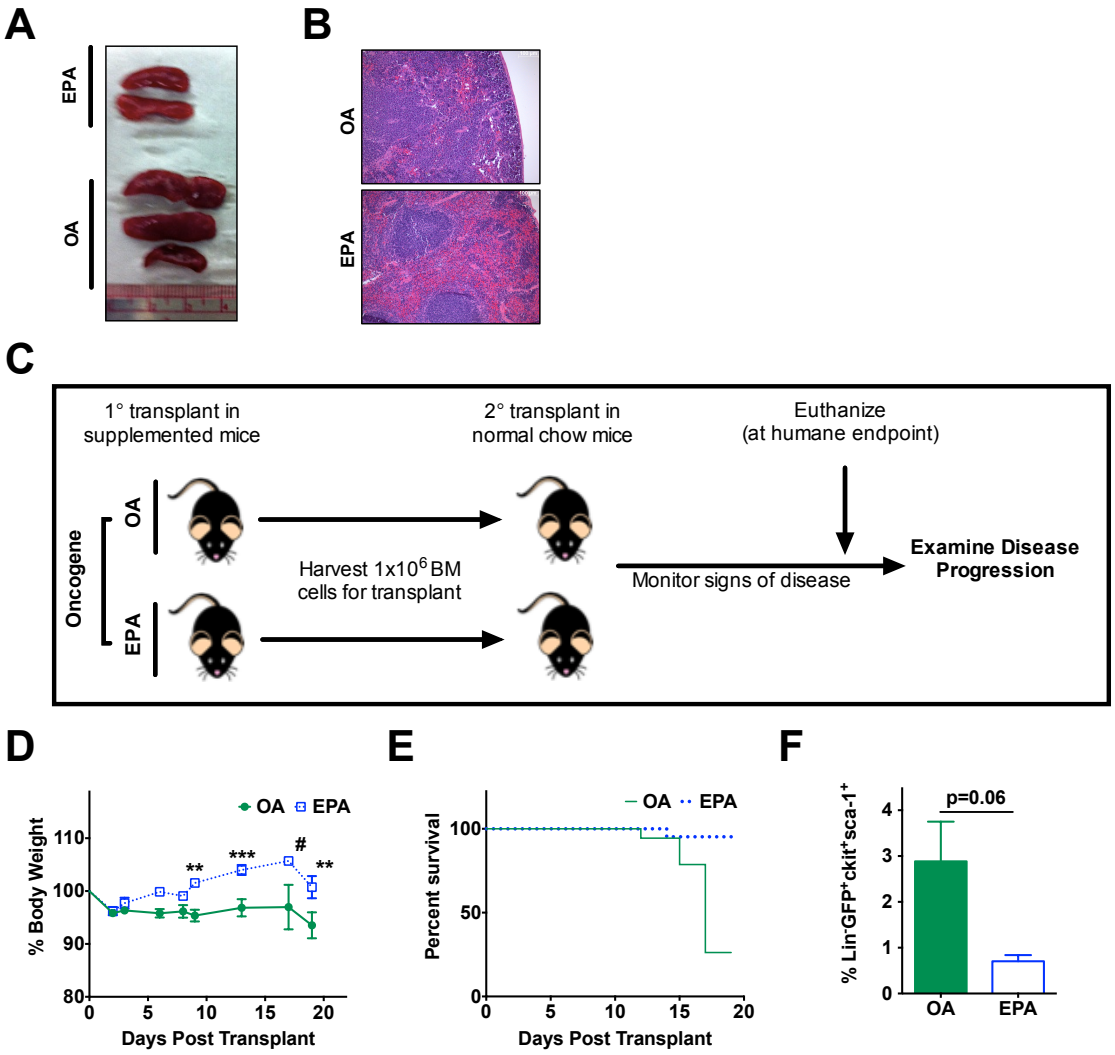

Figure **2-5**: EPA decreases CML in EPA supplemented mice.

A, B: BCR-ABL primary transplant; C: Schematic of secondary transplant model; D-G: T315I BCR-ABL transplanted mice. OA- and EPA-supplemented mice were transplanted with whole bone marrow ($5x10^5$ cells). Mice were followed for survival or euthanized at day 18. **A,** Representative example of spleens from BCR-ABL$^+$ LSC transplanted mice on OA- and EPA- supplemented diet. **B,** Representative example of H&E staining in OA- and EPA- supplemented BCR-ABL$^+$ LSC transplanted mice. **C,** Effect of EPA supplementation in serial transplantation assay. Schematic of experimental design. $1x10^6$ whole leukocytes from bone marrow of EPA- or OA-supplemented BCR-ABL$^+$ LSC transplanted mice (primary transplant) were transplanted into normal chow mice. Mice were euthanized four weeks post-transplant upon signs and symptoms of disease (at humane endpoint). The same protocol was used for secondary AML transplant. **D**, Body weight change, as %, in T315I BCR-ABL transplanted mice. n= 4. **p<0.01, ***p<0.001, #p<0.0001. **E**, Percent survival of T315I BCR-ABL transplanted mice. Experiment end: day 18. n=6. **F**, Flow cytometric analysis of T315I LSCs (Lin$^-$ GFP$^+$c-kit$^+$sca-1$^+$) in OA- and EPA-supplemented mice. n= 2. Unless previously noted, data represent individual mice with error bars of mean ± SEM.

Inhibition of cyclooxygenase and the downstream CyPG cascade blocks the antileukemic effect of EPA

We hypothesized that COX activity was critical in mediating the antileukemic effects of EPA supplementation. To examine such a relationship, we used the NSAID indomethacin, a non-specific inhibitor of COX-1 and COX-2, to limit the endogenous production of prostanoids, including Δ^{12}-PGJ_3. Mice were given indomethacin in drinking water *ad libitum* one week prior to transplantation of BCR-ABL LSCs. Interestingly; the protective property of EPA supplementation in BCR-ABL LSC-transplanted mice was blocked by indomethacin (Fig. 2-6A-E). Indomethacin-treated mice on both OA and EPA diet showed the same severity of disease as measured by the development of splenomegaly, leukocytosis, and disruption of normal splenic architecture, represented by total histology scoring (Fig. 2-6A-C, Fig. 2-7A, B). We previously reported Δ^{12}-PGJ_3 as mediating apoptosis in LSCs (2). To determine the level of apoptosis in EPA-supplemented LSC-transplanted mice, TUNEL staining was preformed on splenic sections. EPA-supplemented BCR-ABL mice displayed increased apoptosis in the spleen compared to OA-supplemented mice (Fig. 2-6D). In contract, indomethacin treatment eliminated the antileukemic property of EPA as measured by the decreased $TUNEL^+$ cells following NSAID treatment. There was no difference in apoptosis between dietary groups in healthy (non-leukemic) splenic tissue (Fig. 2-4D). These data suggest that a metabolite of the COX pathway was responsible for the increased apoptosis, in EPA-supplemented CML mice. Furthermore, flow cytometric analysis of LSCs showed that indomethacin blocked the protective effects of EPA. EPA-supplemented CML mice treated with indomethacin showed no difference in the percentage of LSCs in the spleen and bone marrow when compared to OA alone and OA + indomethacin groups (Fig. 2.6E). However, treatment of EPA-fed mice on indomethacin with exogenous Δ^{12}-PGJ_3 starting one-week post-transplant of LSCs rescued the anti-leukemia effect in these mice as seen by significantly lowered BCR-ABL LSCs in the spleen and bone marrow, which were similar to that observed in EPA-

treated mice (Fig. 2-6E). Surprisingly, the general phenotype of these mice was only partly restored to that of EPA-supplemented mice, as seen by decreased splenomegaly (Fig. 2-7C, D).

To further examine if PGD_3-derived CyPGs mediated the EPA-effect, BCR-ABL mice were given *i.p.* injections of HQL-79, a specific H-PGDS enzyme inhibitor, from time of transplantation to experiment end (Fig. 2-7E). At day 14 post-transplant, HQL-79 treatment of EPA-fed mice had significantly higher total WBCs, as compared to EPA-supplemented mice (Fig. 2-6F, Fig. 2-7F) and displayed similar disease index to OA-supplemented and OA + HQL-79 BCR-ABL mice. The BCR-ABL LSC population was analyzed by flow cytometry and LSC-CFU analysis. HQL-79 treatment of EPA mice doubled the LSC population in the spleen (Fig. 2-7G). BCR-ABL LSC-CFU formation was significantly increased in the EPA + HQL-79 group compared to EPA only. In the bone marrow there was a 6.5 fold increase in LSC-CFU formation, and splenocytes formed 5.5 fold more LSC-CFUs (Fig. 2-6G, H). There was no difference in LSC-CFU formation between OA diet only and OA diet + HQL-79 in BCR-ABL transplanted mice (Fig. 2-6G, H). HQL-79 treatment eliminated the protective effect of EPA supplementation and returned BCR-ABL LSCs to levels as in OA controls. These studies reveal that COX and H-PGDS play an important role in the antileukemic function of EPA in BCR-ABL transplanted mice.

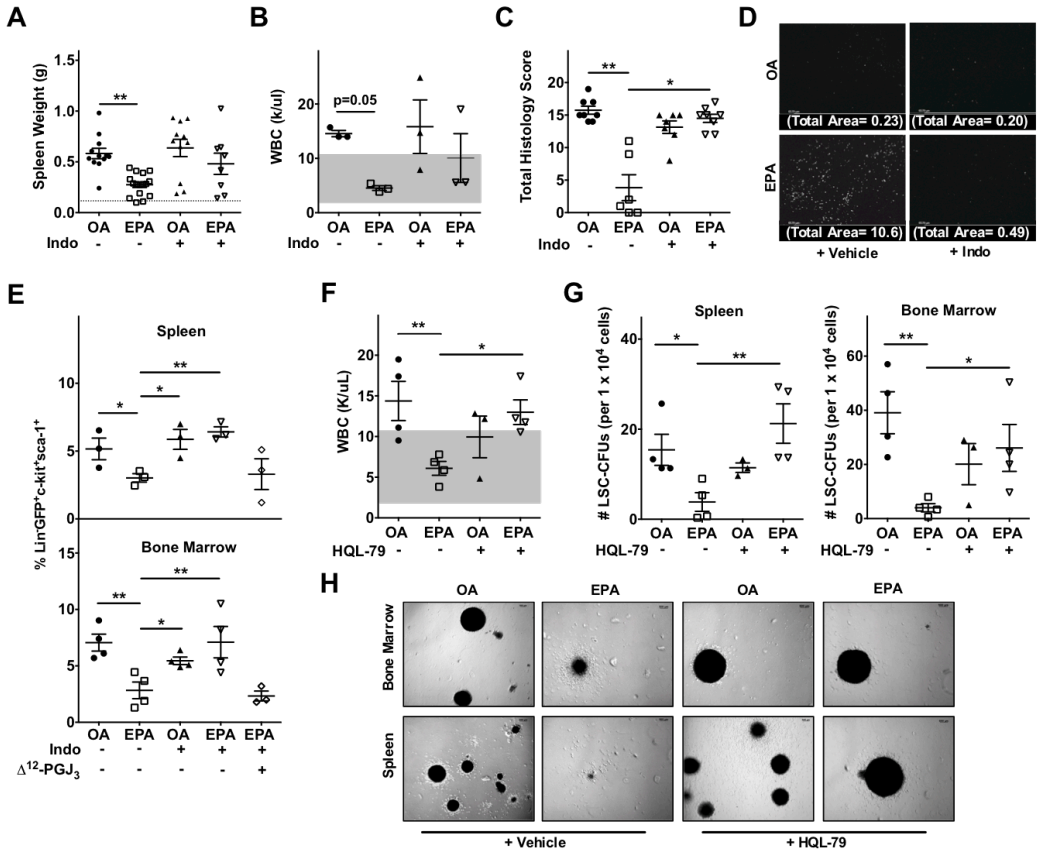

Figure **2-6**: EPA decreases CML in EPA supplemented mice.

A-E: EPA- or OA-supplemented mice were on diet for seven weeks prior to indomethacin (0.00325% w/v). Mice were transplanted with BCR-ABL$^+$ LSCs at week 8; F-H: Mice were irradiated with 450 rads and transplanted with 1x10^6 total BCR-ABL cells. Mice were treated with HQL-79 (30mg/kg) by i.p. injection throughout bone marrow transplant. **A**, Spleen size of EPA- or OA-supplemented mice with or without indomethacin (indo) treatment. Healthy spleen size: small dashed line (0.1g). Data compiled from six independent experiments. n= 8-16 per group. Data were compared to OA only group for analysis. **B**, WBC counts in peripheral blood of BCR-ABL$^+$ LSC transplanted mice (12-14 days post-transplant). Shaded area (1.8-10.7 k/ml) indicates healthy range of WBC counts. n= 3. **C**, Total histology scores of OA- and EPA-supplemented BCR-ABL$^+$ LSC transplanted mice treated with or without indomethacin (0.00325% w/v). Six parameters (DSA, TI, MF, MK, EMH, and necrosis) were scored from 0-4 and total scores are represented. n= 6-8. **D**, TUNEL assay of the splenic sections from BCR–ABL$^+$ LSC transplanted mice on EPA and OA supplemented diets ± indomethacin. Representative images with TUNEL-positive staining are shown. Total area of TUNEL$^+$ staining is shown below each panel. **E**, Flow cytometric analysis of percentage of Lin$^-$ GFP$^+$c-kit$^+$sca-1$^+$ LSCs in spleen and bone marrow of EPA- or OA-supplemented BCR-ABL$^+$ LSC transplanted mice. Mice were treated with or without indomethacin (indo; 0.00325% w/v). i.p. injection of Δ^{12}-PGJ$_3$ (0.025mg/kg body weight) began seven days post-transplant and continued until sacrifice at day 14. Data are representative of four independent experiments (OA/ EPA); two independent experiments (OA/EPA + indo); one independent experiment (Δ^{12}-PGJ$_3$). Overall sample sizes: bone marrow: OA: n=12, EPA n= 9, OA + Indo: n=6, EPA + Indo: n= 5, EPA + Indo + Δ^{12}-PGJ$_3$: n= 3; spleen: OA: n= 12, EPA n= 9, OA + Indo: n= 7, EPA + Indo: n= 5, EPA + Indo + Δ^{12}-PGJ$_3$: n= 3. **F**, WBC counts in peripheral blood of BCR-ABL transplanted mice on day 16 post-transplant. n= 4. **G**, Total CML LSC-CFU counts in spleen and bone marrow from BCR-ABL transplanted mice. n= 4. **H**, Representative images of CML LSC-CFU from BCR-ABL transplanted mice in spleen and bone marrow. Data represent individual mice with error bars of mean ± SEM. *p< 0.05, **p< 0.01.

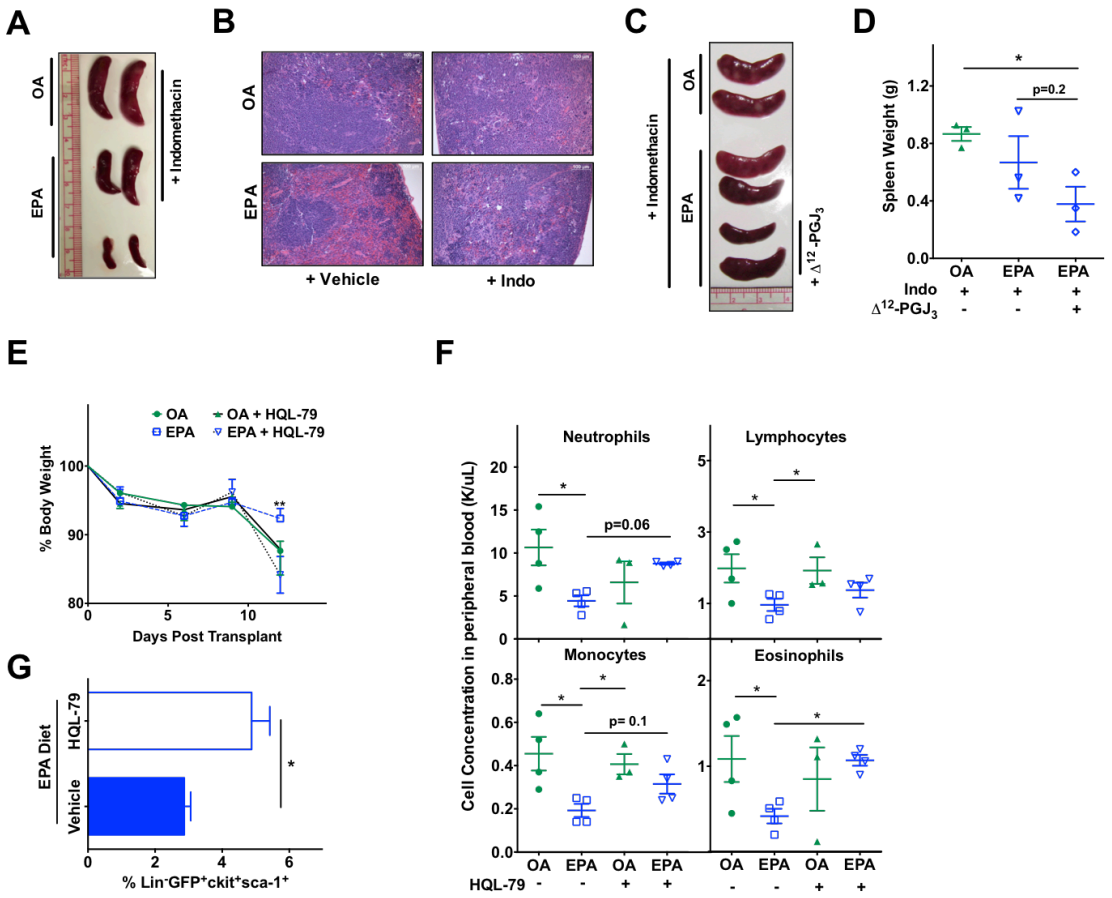

Figure **2-7**: The antileukemic effect of EPA supplementation is dependent on functional production of CyPGs downstream of H-PGDS.

A-D: EPA- or OA- supplemented mice were on diet for seven weeks prior to indomethacin (0.00325% w/v). At 8 weeks, mice were transplanted with BCR-ABL$^+$ LSCs and sacrificed at approximately 14 days post-transplant. Daily i.p. injection of Δ^{12}-PGJ$_3$ (C-D) began 7 days post-transplant and continued until sacrifice at day 14; E-G: OA and EPA supplemented mice transplanted with total BCR-ABL bone marrow (1x10^6 cells). HQL-79 (30mg/kg body weight) was administered by i.p. injection throughout transplant period (total of seven injections). Mice were euthanized on day 14 post-transplant. Representative image of spleen (**A**) and splenic H&E sections (**B**) from BCR-ABL$^+$ LSC transplanted mice OA- and EPA-supplemented mice treated with or without indomethacin (indo), as described. Representative spleen image (**C**) and spleen weight (**D**) in indomethacin treated mice with or without addition of Δ^{12}-PGJ$_3$ treatment. n= 3. **E**, Body weight change, as %, in EPA- and OA-supplemented BCR-ABL$^+$ transplanted mice treated by i.p. as described, with HQL-79. n= 4. **F**, Analysis of peripheral blood composition in BCR-ABL transplanted EPA- and OA-supplemented mice treated with and without HQL-79. n= 4. **G**, Flow cytometric analysis of LSCs in the bone marrow of EPA supplemented BCR-ABL mice treated with and without HQL-79. n= 3. Bar represents mean. Unless previously noted, data represent individual mice with error bars of mean ± SEM. *p<0.05.

EPA supplemented mice display decreased formation of LSC-CFUs in AML

The MLL-AF9 bone marrow transplant model was used to explore whether EPA was protective in AML. At day 13 post transplant, analysis of WBC showed severe leukocytosis in all OA-supplemented mice, while two EPA-supplemented mice had WBC counts within the normal healthy range (Fig. 2-8A). Further analysis of mice showed a slight increase in spleen weight in OA- as compared to EPA-supplemented AML mice (Fig. 2-8B). However, of greatest interest was the effect of EPA on suppressing AML LSC-CFUs. There was a significant decrease in the formation of AML LSC-CFUs from EPA-supplemented spleen and bone marrow. OA splenocytes and bone marrow showed a nearly two-fold increase and a four-fold increase, respectively, compared to EPA (Fig. 2-8C, D). Though EPA colonies were formed, the morphology of these colonies appeared to be CFU-GM like in nature (Fig. 2-8D).

Secondary transplantation with either OA or EPA total bone marrow was used to determine the functionality of AML LSCs in EPA-supplemented mice (see Fig. 2-5C for experimental set-up). Though the peripheral WBC count was not different between diets at 14 days post-transplant, there was a general decrease in WBCs in mice transplanted with EPA-supplemented bone marrow (Fig. 2-8E). There was a significant decrease in the spleen size in secondary transplanted EPA mice (Fig. 2-8F). AML LSC-CFUs were lower in the bone marrow of mice transplanted with EPA-supplemented AML cells, but the differences did not reach significance (Fig. 2-8G, H). In contrast, there was a significant decrease in AML LSC-CFUs from the spleen of secondary EPA-transplanted mice (Fig. 2-8G). Representative examples of AML LSC-CFUs from secondary transplanted mice showed clear differences in the morphology of the colonies (Fig. 2-8H). OA colonies were similar to those from primary transplant, whereas EPA colonies are clearly differentiated from the typical AML LSC-CFU. These results also suggest that the antileukemic effect of EPA supplementation is not limited to a specific oncogene.

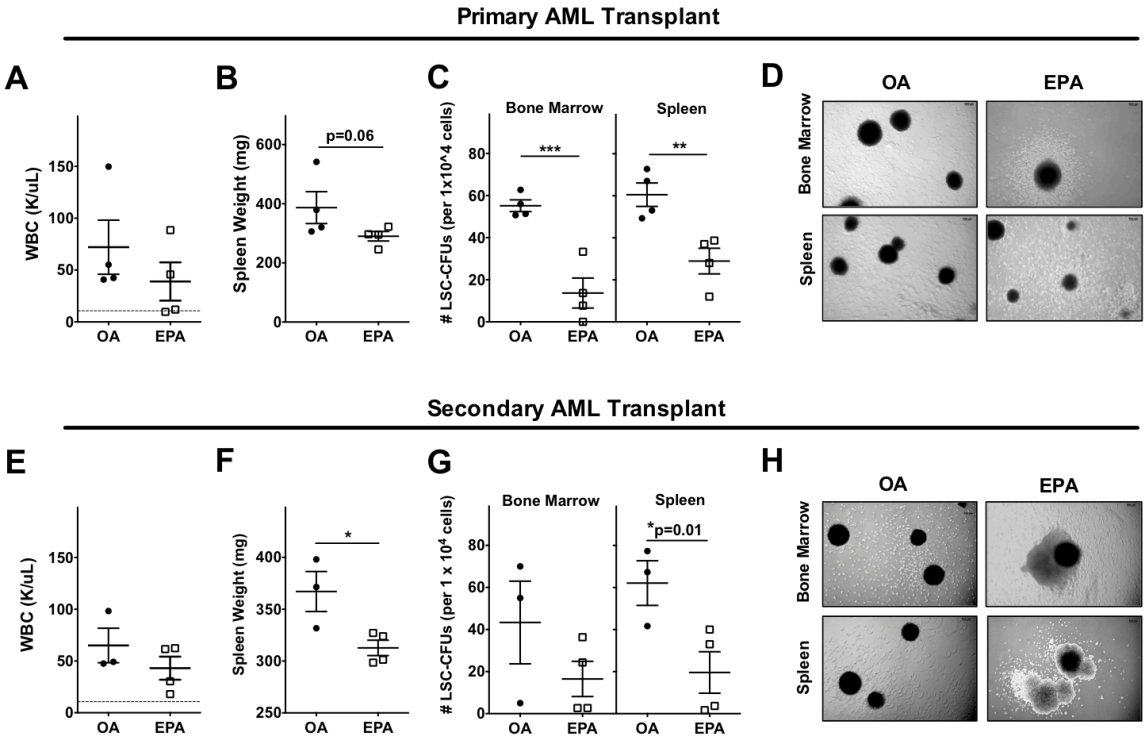

Figure **2-8**: EPA-supplemented mice display decreased signs and symptoms of AML progression.

A-D: Primary AML transplant of $1x10^6$ total AML bone marrow. Mice were euthanized on day 13 post-transplant; E-H: Secondary AML transplant of $1x10^6$ pooled bone marrow from primary OA or EPA supplemented AML mice (Fig. 2-5C for details). Mice were euthanized at day 14 post-transplant. . **A**, WBC counts in peripheral blood of primary OA and EPA supplemented mice. **B**, Spleen weight of primary OA- and EPA-supplemented AML mice. **C**, Total AML LSC-CFU counts in the bone marrow and spleen of primary transplanted OA- and EPA-supplemented mice. **D**, Representative image of AML LSC-CFU in the bone marrow and spleen of primary transplanted OA and EPA supplemented mice. **E**, WBC counts in peripheral blood of secondary transplanted AML mice. **F**, Spleen weight of mice transplanted with AML cells from OA- or EPA-supplemented mice. **G**, Total AML LSC-CFU counts in the bone marrow and spleen of secondary transplanted AML mice. **H**, Representative image of AML LSC-CFU from the bone marrow and spleen of secondary transplanted AML mice. Data represent individual mice with error bars of mean ± SEM. n= 4. *p< 0.05, **p< 0.01, ***p<0.001.

Discussion

This study demonstrates a novel role for EPA at pharmacological levels in three experimental models of myelogenous leukemia, with a focus on BCR-ABL induced CML. To our knowledge, no previous study has investigated the *in vivo* role of EPA supplementation on CML or AML progression. Moreover, previous studies cite the beneficial role of omega-3 in limiting COX activity (34), whereas our data indicate that the activity of COX in EPA-supplemented conditions contributes toward the endogenous production of CyPGs, such as Δ^{12}-PGJ_3. Mice on an EPA-supplemented diet displayed decreased signs and symptoms of leukemia, including a decrease in the LSC population when compared to mice on an OA-supplemented diet. Furthermore, indomethacin and HQL-79 blocked the antileukemic effect of EPA. This supports our hypothesis that EPA supplementation limits CML due to generation of endogenous CyPGs, most importantly those derived downstream of COX-2 and H-PGDS, namely Δ^{12}-PGJ_3. The dose of EPA used in this study was selected specifically to translate to the human pharmaceutical dosage. The FDA approved drug Lovaza®, comprised of 1.86g EPA (55%) and 1.5g DHA (45%), provides an equivalent dosage of EPA as in our study. We limited the supplementation only to EPA to understand the role of EPA-derived CyPGs via the COX-H-PGDS pathway.

We observed a wide range of detectable Δ^{12}-PGJ_3 extracted from the cell culture media and plasma following EPA supplementation. While the trend of EPA leading to increased Δ^{12}-PGJ_3 remained consistent between independent experiments, the total amount of the compound was variable. This could be due to the extractability of Δ^{12}-PGJ_3 and/or the ability of this Michael acceptor electrophile to bind to proteins, thus limiting efficient extraction. In addition, metabolic transformation of Δ^{12}-PGJ_3 could also contribute to the observed variability since such metabolic products were not part of LC-MS/MS-based quantitation.

Although targeting inflammation with NSAID treatment has been an attractive proposition in certain types of cancer, our studies demonstrate a potential detrimental role for NSAID use in CML, which may also be true in AML. Most epidemiological studies, to date, are inconclusive regarding the association between non-aspirin NSAID use and leukemia (12-14). Given the controversial nature of the role of NSAIDs in CML, future studies to associate NSAIDs as a potential confounder in omega-3 PUFA trials may shed more light on the role of endogenous CyPGs.

Current chemotherapies are able to control CML for a period of time, but are unable to cure the disease, as a result of the sustained LSC population (16-18) (19-21, 35). We have previously demonstrated selective targeting of CML LSCs by exogenous treatment with Δ^{12}-PGJ_3 (2). By making a change in dietary composition, we demonstrate that Δ^{12}-PGJ_3 can be synthesized at levels capable of limiting the disease. It is intriguing to note that the effect of EPA of reducing CML was not due to general differences in the ability of these mice to engraft a foreign bone marrow following irradiation. Engraftment and general parameters of health were not different between diets (Fig. 2-4E-I). Thus, suppression of the LSC population was primarily due to dietary manipulation, as confirmed by secondary transplantation of bone marrow from EPA-fed leukemic mice into mice fed on normal chow. These data suggest that dietary supplementation is an attractive way to attenuate the relapse of CML, particularly in TKI resistant CML that only responds to ponatinib, a TKI dispensed with a black box warning. Surprisingly, even in the experimental model of MLL-AF9-induced AML that represents aggressive leukemogenesis, high dose EPA proved to be effective. Thus, based on our studies, it appears that the use of pharmacological doses of EPA in combination with existing standard of care could open new avenues for the successful management of myeloid leukemias.

Our previous work showed that treatment of BCR-ABL LSCs with EPA failed to cause any appreciable increase in apoptosis, supporting the role of the microenvironment in CyPG

production (2). The microenvironment, comprised of immune and non-immune cells, has been shown to be important in understanding cancer outcomes (36, 37). Macrophages have been implicated as being major producers of COX-derived metabolites, including Δ^{12}-PGJ_3 (2). As shown in Fig 2-9, Δ^{12}-PGJ_3 is thought to act through paracrine signaling mechanisms on LSCs to activate pathways of apoptosis; this effect does not appear to be limited to CML alone.

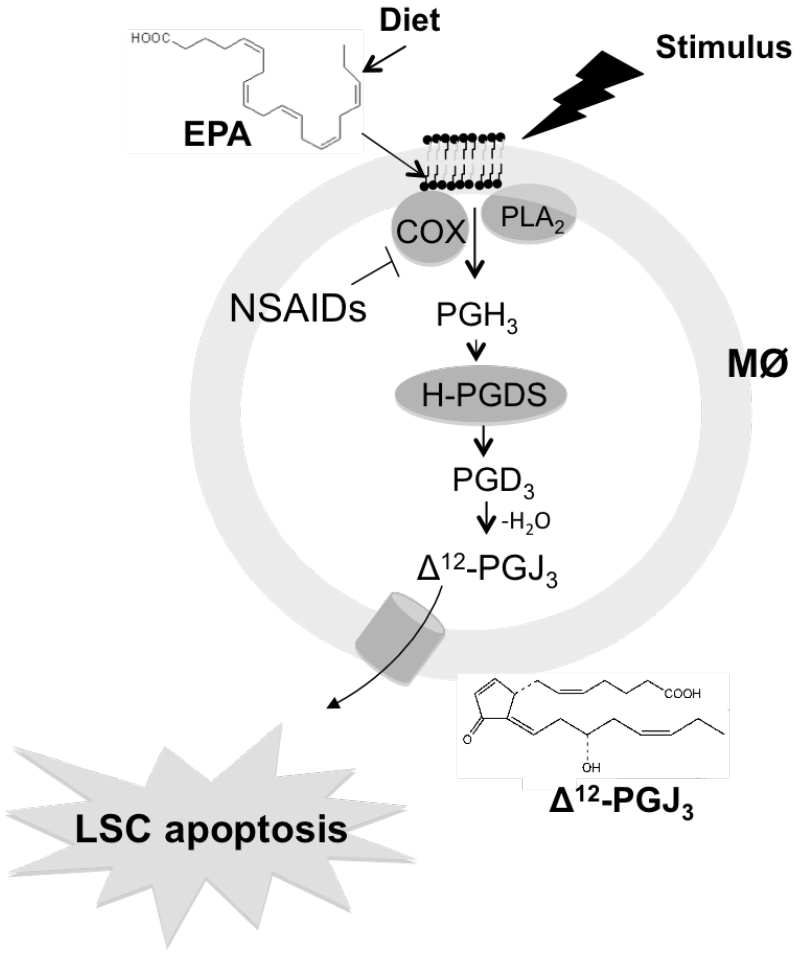

Figure **2-9**: Summary of antileukemic properties of dietary EPA supplementation in myeloid leukemia transplanted mice.

Dietary EPA is incorporated into the phospholipid bilayer of cells. Upon stress, EPA is mobilized by PLA_2 that is acted upon by COX-2 to form PGH_3. NSAIDs, including indomethacin, inhibit the activity of COX, thus down-regulating the production of all downstream mediators. H-PGDS acts on PGH_3 to produce PGD_3. Non-enzymatic dehydration of PGD_3 leads to formation of Δ^{12}-PGJ_3. Inhibition of H-PGDS by HQL-79 blocks the anti-leukemic property observed in EPA-supplemented mice. Δ^{12}-PGJ_3 is transported from the cell (depicted as macrophage) and targets the LSC for apoptosis.

Our data support the pivotal role of COX and H-PGDS in the antileukemic effect of EPA, suggesting that any variation in gene expression and/or activity of either or both of these enzymes could potentially impact the outcome. This observation is not limited to leukemia. In fact, several single nucleotide polymorphisms (SNPs) have been identified in the COX-2 gene, *PTGS2,* and meta-analysis has suggested a possible association to breast cancer (38). Therefore, the translatability of these studies into human therapy would depend on the effect the SNPs have on the expression and/or activity of COX and H-PGDS. Few genome-wide association studies have explored SNPs in the COX gene and resulting leukemia outcomes (39), highlighting an area that should be more closely studied in the future.

In summary, our studies use well-established rodent models of CML, T315I CML, and MLL-AF9 AML to demonstrate that dietary EPA decreases the severity of disease. In the case of CML, the antileukemic effect of EPA was blocked by both indomethacin and HQL-79, therefore confirming the essential role of the COX pathway in the metabolism of EPA into a novel class of endogenous CyPGs, including Δ^{12}-PGJ_3. Although deemed safe, the success of this therapy rests on the efficient metabolism of EPA to endogenous levels of CyPGs that target LSCs. Future clinical trials with EPA are warranted to conclusively provide a compelling case for its use as an adjuvant therapy in myeloid leukemia patients.

References

1. Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010) Cancer statistics, 2010. *CA: a cancer journal for clinicians* **60**, 277-300
2. Hegde, S., Kaushal, N., Ravindra, K. C., Chiaro, C., Hafer, K. T., Gandhi, U. H., Thompson, J. T., van den Heuvel, J. P., Kennett, M. J., Hankey, P., Paulson, R. F., and Prabhu, K. S. (2011) Δ12-prostaglandin J3, an omega-3 fatty acid-derived metabolite, selectively ablates leukemia stem cells in mice. *Blood* **118**, 6909-6919
3. Calder, P. C., and Grimble, R. F. (2002) Polyunsaturated fatty acids, inflammation and immunity. *European journal of clinical nutrition* **56 Suppl 3**, 9
4. Greene, E. R., Huang, S., Serhan, C. N., and Panigrahy, D. (2011) Regulation of inflammation in cancer by eicosanoids. *Prostaglandins & other lipid mediators* **96**, 27-36
5. Wall, R., Ross, R. P., Fitzgerald, G. F., and Stanton, C. (2010) Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutrition reviews* **68**, 280-289
6. Wallace, J. M. (2002) Nutritional and botanical modulation of the inflammatory cascade—eicosanoids, cyclooxygenases, and lipoxygenases—as an adjunct in cancer therapy. *Integrative Cancer Therapies* **1**, 7-37
7. Serhan, C. N. (2014) Pro-resolving lipid mediators are leads for resolution physiology. *Nature* **510**, 92-101
8. Morita, M., Kuba, K., Ichikawa, A., Nakayama, M., Katahira, J., Iwamoto, R., Watanebe, T., Sakabe, S., Daidoji, T., Nakamura, S., Kadowaki, A., Ohto, T., Nakanishi, H., Taguchi, R., Nakaya, T., Murakami, M., Yoneda, Y., Arai, H., Kawaoka, Y., Penninger, J. M., Arita, M., and Imai, Y. (2013) The lipid mediator protectin D1 inhibits influenza virus replication and improves severe influenza. *Cell* **153**, 112-125
9. Morris, T., Rajakariar, R., Stables, M., and Gilroy, D. W. (2006) Not all eicosanoids are bad. *Trends in pharmacological sciences* **27**, 609-611
10. Vane, J. R., Bakhle, Y. S., and Botting, R. M. (1998) Cyclooxygenases 1 and 2. *Annual review of pharmacology and toxicology* **38**, 97-120
11. Wada, M., DeLong, C. J., Hong, Y. H., Rieke, C. J., Song, I., Sidhu, R. S., Yuan, C., Warnock, M., Schmaier, A. H., Yokoyama, C., Smyth, E. M., Wilson, S. J., FitzGerald, G. A., Garavito, R. M., Sui, D. X. e. X., Regan, J. W., and Smith, W. L. (2007) Enzymes and receptors of prostaglandin pathways with arachidonic acid-derived versus eicosapentaenoic acid-derived substrates and products. *The Journal of biological chemistry* **282**, 22254-22266
12. Kasum, C. M., Blair, C. K., Folsom, A. R., and Ross, J. A. (2003) Non-steroidal anti-inflammatory drug use and risk of adult leukemia. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **12**, 534-537
13. Ross, J. A., Blair, C. K., Cerhan, J. R., Soler, J. T., Hirsch, B. A., Roesler, M. A., Higgins, R. R., and Nguyen, P. L. (2011) Nonsteroidal anti-inflammatory drug and acetaminophen use and risk of adult myeloid leukemia. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **20**, 1741-1750
14. Rohrbacher, M., and Hasford, J. (2009) Epidemiology of chronic myeloid leukaemia (CML). *Best practice & research. Clinical haematology* **22**, 295-302

15. Kimura, S., Ando, T., and Kojima, K. (2014) Ever-advancing chronic myeloid leukemia treatment. *International journal of clinical oncology* **19**, 3-9
16. Hamad, A., Sahli, Z., El Sabban, M., Mouteirik, M., and Nasr, R. (2013) Emerging therapeutic strategies for targeting chronic myeloid leukemia stem cells. *Stem cells international* **2013**, 724360
17. Rea, D., Rousselot, P., Guilhot, J., Guilhot, F., and Mahon, F.-X. X. (2012) Curing chronic myeloid leukemia. *Current hematologic malignancy reports* **7**, 103-108
18. Jain, P., Kantarjian, H., and Cortes, J. (2013) Chronic myeloid leukemia: overview of new agents and comparative analysis. *Current treatment options in oncology* **14**, 127-143
19. Ernst, T., and Hochhaus, A. (2012) Chronic myeloid leukemia: clinical impact of BCR-ABL1 mutations and other lesions associated with disease progression. *Seminars in oncology* **39**, 58-66
20. Hochhaus, A., Ernst, T., Eigendorff, E., and La Rosée, P. (2015) Causes of resistance and treatment choices of second- and third-line treatment in chronic myelogenous leukemia patients. *Annals of hematology* **94 Suppl 2**, 40
21. Horton, S. J., Jaques, J., Woolthuis, C., van Dijk, J., Mesuraca, M., Huls, G., Morrone, G., Vellenga, E., and Schuringa, J. J. (2013) MLL-AF9-mediated immortalization of human hematopoietic cells along different lineages changes during ontogeny. *Leukemia* **27**, 1116-1126
22. Weldon, K. A., and Whelan, J. (2011) Allometric scaling of dietary linoleic acid on changes in tissue arachidonic acid using human equivalent diets in mice. *Nutrition & metabolism* **8**, 43
23. Gandhi, U. H., Kaushal, N., Ravindra, K. C., Hegde, S., Nelson, S. M., Narayan, V., Vunta, H., Paulson, R. F., and Prabhu, K. S. (2011) Selenoprotein-dependent up-regulation of hematopoietic prostaglandin D2 synthase in macrophages is mediated through the activation of peroxisome proliferator-activated receptor (PPAR) gamma. *The Journal of biological chemistry* **286**, 27471-27482
24. Vunta, H., Belda, B. J., Arner, R. J., Channa Reddy, C., Vanden Heuvel, J. P., and Sandeep Prabhu, K. (2008) Selenium attenuates pro-inflammatory gene expression in macrophages. *Molecular nutrition & food research* **52**, 1316-1323
25. Kudva, A. K., Kaushal, N., Mohinta, S., Kennett, M. J., August, A., Paulson, R. F., and Prabhu, K. S. (2013) Evaluation of the stability, bioavailability, and hypersensitivity of the omega-3 derived anti-leukemic prostaglandin: Δ(12)-prostaglandin J3. *PloS one* **8**
26. Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L., and Baltimore, D. (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* **92**, 3780-3792
27. Schaefer, B. C., Schaefer, M. L., Kappler, J. W., Marrack, P., and Kedl, R. M. (2001) Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions in vivo. *Cellular immunology* **214**, 110-122
28. Gandhi, U. H., Kaushal, N., Hegde, S., Finch, E. R., Kudva, A. K., Kennett, M. J., Jordan, C. T., Paulson, R. F., and Prabhu, K. S. (2014) Selenium suppresses leukemia through the action of endogenous eicosanoids. *Cancer research* **74**, 3890-3901
29. Hashimoto, K., Sheller, J. R., Morrow, J. D., Collins, R. D., Goleniewska, K., O'Neal, J., Zhou, W., Ji, S., Mitchell, D. B., Graham, B. S., and Peebles, R. S. (2005) Cyclooxygenase inhibition augments allergic inflammation through CD4-dependent, STAT6-independent mechanisms. *Journal of immunology (Baltimore, Md. : 1950)* **174**, 525-532

30. Pessina, A., Albella, B., Bueren, J., Brantom, P., Casati, S., Gribaldo, L., Croera, C., Gagliardi, G., Foti, P., Parchment, R., Parent-Massin, D., Sibiril, Y., and Van Den Heuvel, R. (2001) Prevalidation of a model for predicting acute neutropenia by colony forming unit granulocyte/macrophage (CFU-GM) assay. *Toxicology in vitro : an international journal published in association with BIBRA* **15**, 729-740
31. Palozza, P., Sgarlata, E., Luberto, C., Piccioni, E., Anti, M., Marra, G., Armelao, F., Franceschelli, P., and Bartoli, G. M. (1996) n-3 fatty acids induce oxidative modifications in human erythrocytes depending on dose and duration of dietary supplementation. *The American journal of clinical nutrition* **64**, 297-304
32. Wertheim, J. A., Miller, J. P., Xu, L., He, Y., and Pear, W. S. (2002) The biology of chronic myelogenous leukemia:mouse models and cell adhesion. *Oncogene* **21**, 8612-8628
33. Neering, S. J., Bushnell, T., Sozer, S., Ashton, J., Rossi, R. M., Wang, P.-Y. Y., Bell, D. R., Heinrich, D., Bottaro, A., and Jordan, C. T. (2007) Leukemia stem cells in a genetically defined murine model of blast-crisis CML. *Blood* **110**, 2578-2585
34. Berquin, I. M., Edwards, I. J., and Chen, Y. Q. (2008) Multi-targeted therapy of cancer by omega-3 fatty acids. *Cancer Letters* **269**, 363-377
35. Crews, L. A., and Jamieson, C. H. (2012) Chronic myeloid leukemia stem cell biology. *Current hematologic malignancy reports* **7**, 125-132
36. Brglez, V., Lambeau, G., and Petan, T. (2014) Secreted phospholipases A2 in cancer: Diverse mechanisms of action. *Biochimie* **107PA**, 114-123
37. Crews, L. A., and Jamieson, C. H. (2013) Selective elimination of leukemia stem cells: hitting a moving target. *Cancer letters* **338**, 15-22
38. Dai, Z.-J. J., Shao, Y.-P. P., Ma, X.-B. B., Xu, D., Tang, W., Kang, H.-F. F., Lin, S., Wang, M., Ren, H.-T. T., and Wang, X.-J. J. (2014) Association of the three common SNPs of cyclooxygenase-2 gene (rs20417, rs689466, and rs5275) with the susceptibility of breast cancer: an updated meta-analysis involving 34,590 subjects. *Disease markers* **2014**, 484729
39. Wang, C.-H. H., Wu, K.-H. H., Yang, Y.-L. L., Peng, C.-T. T., Wang, R.-F. F., Tsai, C.-W. W., Tsai, R.-Y. Y., Lin, D.-T. T., Tsai, F.-J. J., and Bau, D.-T. T. (2010) Association study of cyclooxygenase 2 single nucleotide polymorphisms and childhood acute lymphoblastic leukemia in Taiwan. *Anticancer research* **30**, 3649-3653

Chapter 3

Antileukemic effect of selenium supplementation is mediated through endogenous activation of peroxisome activated receptor gamma (PPARγ)

Emily R. Finch, Diwakar B. Tukaramrao, Laura L. Goodfield, Michael D. Quickel, Robert F. Paulson, K. Sandeep Prabhu

Department of Veterinary and Biomedical Sciences, Center for Molecular Immunology and Infectious Diseases and Center for Molecular Toxicology and Carcinogenesis, 115 Henning Building, The Pennsylvania State University, University Park, PA 16802

Contributions: Emily R. Finch made all figures conducted all experiments. LC-MS/MS samples were run by Diwakar B. Tukaramrao. Laura L. Goodfield aided in sample collection. Michael D. Quickel prepared retrovirus.

Abstract

Micronutrient selenium supplementation in chronic myelogenous mice (CML) mice alleviates leukemia via the elimination of leukemia stem cells (LSCs). Use of NSAIDs has suggested that such a phenomenon is mediated through the upregulation of endogenous cyclopentenone prostaglandins (CyPGs), namely delta-12 prostaglandin J2 (Δ^{12}-PGJ_2) and 15-deoxy-delta 12,14- prostaglandin J_2 (15d-PGJ_2). In this report, we show that these endogenous CyPGs alleviate symptoms of CML through their ability to activate the nuclear hormone receptor, peroxisome proliferator activated receptor gamma (PPARγ). Supplementation of CML mice with supraphysiological, but non-toxic doses, of selenium (at 0.4 ppm) largely eradicated signs and symptoms of the disease, with low traces of LSCs. However, use of GW9662, a PPARγ antagonist, in selenium supplemented CML mice blocked the anti-leukemic effect of selenium. Treatment of LSCs with selenium in the presence of GW9662 increased the activation of STAT5 and a downstream master regulator of LSC quiescence, CITED2. GW9662 treatment also increased the expression of 15- prostaglandin dehydrogenase (15-PGDH) that oxidizes many bioactive lipids, including 15d-PGJ_2 and Δ^{12}-PGJ_2 and, thus, abrogated the protective effect of selenium. These studies suggest a potential role for selenium supplementation as an adjuvant therapy in CML.

Introduction

Chronic myeloid leukemia (CML) is a cancer that is associated with an abnormal increase in non-functioning myeloid-derived white blood cells (WBCs). The disease is characterized by a reciprocal chromosomal translocation between the *abl* gene on chromosome 9 and the *bcr* gene on chromosome 22 (t(9;22)(q34;q11)) to form the Philadelphia chromosome (1-4). As a result, a fusion oncoprotein, BCR-ABL, is formed that is endowed with a deregulated tyrosine kinase activity, leading to uncontrolled proliferation (including auto-phosphorylation), loss of stromal adhesion (5), and resistance to apoptosis (6). The fusion tyrosine kinase is the target of current therapeutics (1, 2) such as imatinib and other new generation tyrosine kinase inhibitors (TKIs) (7).

The chemoresistant cells in CML are the quiescent, self-renewing leukemia stem cell population (LSC) (8-10) (11). BCR-ABL activity is not absolutely essential for the survival of LSCs (12). That said the progression of chronic phase CML to blast crisis is partially attributed to acquisition of a new LSC population. Thus, targeting of pathways involved in the maintenance of LSCs is suggested to serve as potential therapeutic targets (13-15). A recent study indicated that actively targeting of PPARγ in patient-derived CML LSCs made the cells more sensitive to imatinib (16).

It is well established that the nuclear receptor PPARγ is a central regulator of metabolism and cellular replication. PPARγ is activated by synthetic agonists, such as thiazolidinediones (TZDs; rosiglitazone and pioglitazone), and endogenous metabolites, such as cyclopentenone prostaglandins (CyPGs), including 15-deoxy-delta 12,14- prostaglandin J_2 (15d-PGJ_2) and delta-12 prostaglandin J2 (Δ^{12}-PGJ_2) (17, 18). Interestingly, *in vitro* studies revealed a potential antileukemic effect of PPARγ ligands (19, 20) with a focus on 15d-PGJ_2 and TZDs.

We have previously shown selenium supplementation in macrophages to cause eicosanoid class switching in an inflammatory setting (18, 21). Selenium increased the expression of hematopoietic-prostaglandin D synthase (H-PGDS) (18) leading to the production of Δ^{12}-PGJ_2 and 15d-PGJ_2. Activation of PPARγ by these endogenous ligands led to the transcriptional upregulation of H-PGDS expression to effect a feed forward loop to increase the endogenous levels of Δ^{12}-PGJ_2 and 15d-PGJ_2 (18). In addition, the activity of NF-kB was attenuated resulting in the decreased expression of prostaglandin E synthase (PTGES) and thromboxane A synthase 1 (TXAS1) and their products, PGE_2 and TXA_2, respectively (18, 22). As a result, selenium effectively shunted the eicosanoid pathway from pro-inflammatory PGs towards anti-inflammatory CyPGs. Interestingly, we have shown that the anti-leukemic activity of selenium greatly relied upon a shift in eicosanoid production in models of experimental leukemia (18, 23). The LSC population was significantly decreased in selenium-supplemented mice (23). Based on recent studies (16, 24), we hypothesized that PPARγ activation could play a central role in the protective effect of selenium-supplementation in leukemia, while inhibition of PPARγ could reverse the anti-leukemic effect of selenium.

Prost et al. (16) demonstrated pioglitazone significantly reduced LSC burden and increased susceptibility to imatinib treatment in otherwise resistant LSCs. Glodkowska et al. (24) investigated the role of second and third generation TKIs in combination with PPARγ agonists and saw similar promising results. Activation of PPARγ with pioglitazone decreased STAT5a activation, which is indispensable in maintenance of LSCs (25) in primary BCR-ABL^+ CML samples (16) affecting the two downstream targets CITED2 (CBP/p300-interacting-transactivator with Glu/Asp-rich carboxy-terminal domain 2) and hypoxia-induced transcription factor 2a (HIF2α) that are both essential in maintaining the quiescent population of LSCs.

Here we report the chemopreventative role of selenium supplementation in BCR-ABL mice as being mediated through activation of PPARγ. Activation of PPARγ in LSCs leads to cellular demise. To relate the underlying role of PPARγ in the Se-S CML mice, we used pioglitazone treatment in Se-A mice, as well as inhibited PPARγ in Se-S mice. Our studies indicated that PPARγ was absolutely essential to mediate the anti-leukemic effect of selenium supplementation. 15d-PGJ_2 and Δ^{12}-PGJ_2 were decreased in BCR-ABL mice on Se-S, diet treated with a PPARγ antagonist. On the other hand, abrogation of PPARγ activation in BCR-ABL mice maintained on a Se-S diet increased the activation of STAT5a and its downstream targets, HIF2α and CITED2, genes essential in maintaining LSCs. Taken together, our findings support dietary manipulation with supraphysiological levels of selenium, which increase endogenous PPARγ ligands, may increase the efficacy of standard CML therapies.

Materials and Methods

Selenium diet and mice

Three-week-old C57BL/6 mice (Taconic Biosciences, Hudson, NY) were randomly placed on selenium adequate (0.08 ppm Se as selenite; Se-A) or selenium supplemented (0.4 ppm Se as selenite; Se-S) semi-purified diets (AIN76A; Harlan Teklad, WI) for at least eight weeks. Diets were provided *ad libitum* and mice were maintained on Milli-Q™ water. Mice were housed three to four per cage in a temperature- and humidity-controlled room with a 12 hour light-dark cycle. All mice were 11-16 weeks of age at sacrifice. Mice over 40 grams at time of bone marrow transplant were excluded. The Institutional Animal Use and Care Committee (IACUC) at The Pennsylvania State University preapproved all procedures.

Extraction and LC MS/MS analysis of PGs from serum

Prostaglandins were extracted from serum samples using C-18 Sep-pak cartridge (Waters, Milford, MA). Briefly, serum samples were acidified with 2 N HCl and loaded on to the Sep-Pak cartridge, bound PGs were eluted with methanol, evaporated, and stored in ethyl acetate, until analysed at -80˚C.

After extraction, PGs were suspended in 70% methanol and separated on a Shimadzu HPLC system equipped with an auto sampler, binary pump (LC-20AD), and Phenomenex C-18 Column (150 X 2.0 mm, 3µm), using 70% methanol as mobile phase at a flow rate of 0.15 mL/min. MS/MS detection was performed using an API 2000 LC/MS/MS system with Turbo V source and an electrospray ionization probe, operated in negative ion mode. Quantitative analysis of Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2, and 13,14-dihydro-15-keto-PGJ_2, was performed using calibration curve generated by respective standards under multiple reaction monitoring (MRM) mode. Three transitions were used to detect each PG: Δ^{12}-PGJ_2 (332.8/188.9; 332.8/314.9;

332.8/271.3 m/z), 15-deoxy-Δ^{12},14-PGJ_2 (315.1/271.1; 315.1/203.0; 315.1/158.0 m/z), 13,14-dihydro-15-keto-PGJ_2 (333.2/175.0; 333.2/207.2; 333.2/315.0 m/z). Data acquisition and analysis was performed using Analyst software, version 1.5.

Generation of experimental myeloid leukemia

CML model was generated as previously described (26). Retroviral stocks were generated by transfecting HEK293T cells with pMIG-BCR-ABL (a gift from Dr. Warren Pear, University of Pennsylvania, Philadelphia) and pEco for packaging using TransIT 293 reagent (Mirus Bio, Madison, WI). Transduction of HSCs was modified from previously described (27, 28). Bone marrow cells were transduced with retrovirus; allowed to rest in conditioned media (described below); and transplanted by retro-orbital injection ($1x10^6$-$2x10^6$ trypan blue counted cells) into lethally irradiated mice. Transduced cells were passaged through mice at least two times prior to transplantation into experimental mice. $5x10^5$ BCR-ABL Lin^- cells, isolated using EasySep™ mouse hematopoietic progenitor cell enrichment kit (Stem Cell Technologies, Vancouver, BC) were transplanted by retro-orbital injection into sub-lethally irradiated (475 rads) recipient mice. Disease progression was monitored by complete blood count (CBC) analysis, evaluating peripheral blood for leukocytosis on a Hemavet 950FS equipped with a veterinary software program. Mice were sacrificed between day 14 through day 21 post-transplant, based on disease progression and humane endpoints.

Modulation of PPARγ activity in vivo

PPARγ agonist pioglitazone (5mg/kg body weight) and antagonist GW9662 (1mg/kg body weight) were purchased from Cayman Chemical (Ann Arbor, MI) and dissolved in a solution of hydroxypropyl-β-cyclodextrin (HPBCD; 30mM in sterile water; Sigma Aldrich, St Louis, MO) and administered by *i.p.* injection daily, starting one day prior to BCR-ABL transplant, until mice were euthanized (~ 21 days). HPBCD in sterile water was used as the vehicle control.

Flow cytometry of CML LSCs

Flow cytometry was performed as previously described (26). Whole spleen and bone marrow were collected and red blood cells (RBCs) were lysed with ACK lysis buffer (155 mM NH_4Cl, 12 mM $KHCO_3$, 0.1mnM EDTA-2Na). Isolated Lin^- cells were stained for Ly-6A/E (sca-1) and CD117 (c-kit) (BD Biosciences, San Jose, CA). Lin^- white blood cells (WBCs) and the presence of LSCs were analyzed on Accuri C6 (BD Biosciences, San Jose, CA).

Protein analysis

Lin^- BCR-ABL cell lysate was isolated with M-PER (ThermoFisher Scientific, Waltham, MA). Protein concentration was estimated using the BCA protein assay kit (ThermoFisher Scientific, Waltham, MA). Western immunoblotting analysis was performed by loading equal amount of protein on an SDS-page gel 10 % SDS for protein >70 kDa; 12 % SDS for protein <70 kDa) followed by electroblotting onto PVDF membranes. Membranes were incubated with the following antibodies: 15-prostaglandin dehydrogenase (15-PGDH), thromboxane A synthase (TXAS), and PTGES (prostaglandin E synthase) (all from Cayman Chemical, Ann Arbor, MI), phospho-STAT5 and STAT5 (Cell Signaling Technologies, Danvers, MA), CITED2 (Abcam, Cambridge, UK), HIF2α (R&D Systems, Minneapolis, MN), and GAPDH (Fitzgerald Industries, Acton MA). Experiments were performed in at least biological triplicate and technical duplicate. Autoradiographs were subjected to densitometry analysis by Image J software (National Institutes of Health, Bethesda, MD).

Gene expression analysis

Quantitative RT-PCR (qPCR) with Taqman probes (Life Technologies, Calsbad, CA) was performed to examine gene expression in Lin^- BCR-ABL cells. Data were analyzed according to the method of Livak and Schmittgen with normalization to 18S rRNA (29). For *in vivo* experiments, Se-A pioglitazone BCR-ABL (Fig 3-1) or Se-S vehicle BCR-ABL (Fig 3-2, 3-

5) was used as control, where appropriate. This was done to keep the activation of PPARγ as the control.

LSC-Colony Forming Units (LSC-CFU)

LSC-CFUs were plated and counted as previously described (26). Total splenocytes or bone marrow from leukemic mice was plated in M3231 Methocult (Stem Cell Technologies, Vancouver, British Columbia). Media was supplemented with IL-3 (2.5ng/ml; R&D Systems, Minneapolis, MN), stem cell factor (SCF; 50ng/ml; Gold Biotechnology, St. Louis, MO), growth differentiation factor 15 (GDF-15; 30ng/ml; Biomatik, Wilmington, DE), and sonic hedgehog (Shh; 25ng/ml; Gold Biotechnology, St. Louis, MO). On days 7-10, colonies were counted. LSC-CFU were defined based on their size (large: >100 cells at high magnification), shape (perfectly circular), density (dark brown/black color), and edge (clearly defined edge with limited differentiation). Any variation from LSC-CFU formation was also considered: in some cases granulocyte-macrophage progenitor colony forming units (CFU-GM) were observed (30). Each biological sample was plated in triplicate. The average number of LSC colonies was calculated for each biological replicate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Unless noted, nonparametric tests, including one-way ANOVA and un-paired two-tailed student t-test, were used where appropriate. *In vivo*, all groups had at least four mice per individual experiment, with at least two independent experiments. For BCR-ABL transplantation studies, at least two independent experiments were conducted. Data were compiled from these experiments for a total biological n-value described in figure legends. For technical analysis (flow cytometry, qPCR, and Western blot) samples were run in at least duplicate. The mean of the technical replicates was considered a biological n-value for statistical analyses. PPARγ activation was considered as “baseline” for experiments: Se-A pioglitazone or

Se-S vehicle were used as controls, unless otherwise noted, for t-tests. For ANOVA, all groups were compared, unless noted. Variation from these analyses is described in figure legends. Bars represent biological mean ± SEM: *$p<0.05$, **$p<0.01$, ***$p<0.001$, #$p<0.0001$.

Results

Pioglitazone decreases murine BCR-ABL CML disease burden in Se-adequate mice

To investigate whether PPARγ activation alone, without the addition of TKIs, could rescue these mice, pioglitazone treatment was administered. PPARγ activation in Se-A BCR-ABL mice was confirmed by qPCR analysis. Two PPARγ targets, mannose-receptor C type 1 (Mrc1) and CD68, were increased with pioglitazone treatment (Fig 3-1A), which demonstrated that pioglitazone was effective as a PPARγ agonist. Furthermore, treatment of Se-A BCR-ABL mice with pioglitazone decreased the overall burden of the disease (Fig 3-1B-E). Most importantly, pioglitazone treatment had no effect in healthy mice in terms of WBC counts (Fig 3-1B). The difference in total WBC counts was not significant between Se-A BCR-ABL vehicle and pioglitazone treated mice. However, all Se-A pioglitazone treated BCR-ABL mice had WBC counts in the normal range, whereas Se-A vehicle BCR-ABL mice showed an increased trend in WBCs above the normal range (Fig 3-1B). GFP^+ cells in peripheral blood indicated differences, as GFP is co-expressed with BCR-ABL (27). There was a significant decrease in total peripheral blood GFP in Se-A pioglitazone treated BCR-ABL mice as compared to vehicle treated (Fig 3-1C). In addition, splenomegaly, which is a commonly observed feature of murine BCR-ABL CML (31), was also decreased upon treatment of BCR-ABL Se-A mice with pioglitazone (Fig 3-1D). However, the spleen size was not affected by pioglitazone in healthy Se-A mice (Fig 3-1D). More importantly, the LSC population (defined as $GFP^+Kit^+Sca1^+Lin^-$ cells) was significantly decreased with pioglitazone treatment both in the spleen and bone marrow of Se-A pioglitazone treated mice (Fig 3-1E). Overall, these findings supported the notion that PPARγ activation with pioglitazone treatment was protective in experimental BCR-ABL CML in otherwise susceptible Se-A mice. More importantly, pioglitazone-induced activation of PPARγ decreased the LSC

population to levels similar to that in Se-S mice, even without the addition of TKI treatment (23).

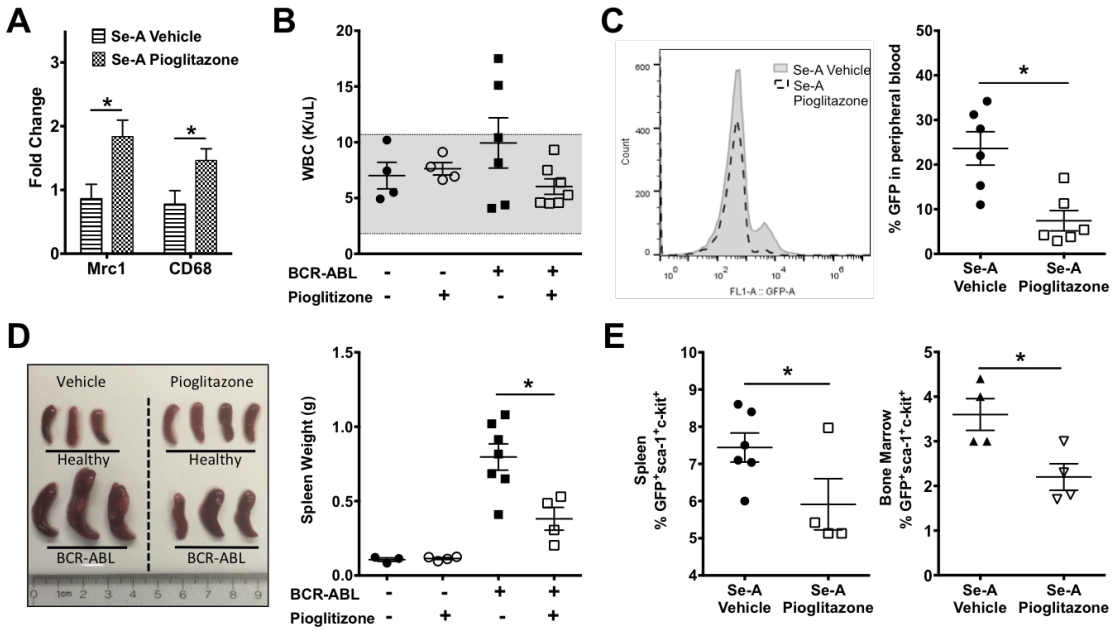

Figure **3-1**: Pioglitazone activation of PPARγ reduces CML burden in Se-A mice.

Pioglitazone (5mg/kg body weight) was given to Se-A mice, on the diet for at least 8 weeks, by *i.p.* injection daily starting one day prior to BCR-ABL transplant, until euthanized. The same duration of treatment was given for healthy pioglitazone mice. Peripheral blood, spleen, and bone marrow were used to measure disease burden. LSC counts were determined from bone marrow and spleen A) Conformation of PPARγ activation with pioglitazone treatment. qPCR expression as fold change in Se-A BCR-ABL splenocytes. Gene expression was normalized to 18s rRNA and Se-A vehicle samples were used as control. n=5. B) Total WBC (K/µl blood) count in peripheral blood of healthy (n=4) and BCR-ABL (n=7) transplanted mice. The shaded box indicates the healthy range. C) Peripheral blood GFP expressed as percentage of gated input in Se-A BCR-ABL transplanted mice (500,000 events collected, gated on FSC). Representative histogram is shown along with individual data points. n=6. D) Representative image and spleen weight in healthy (n=3-4) and BCR-ABL transplanted (n=4-7) mice. E) LSC population evaluation by flow cytometry. LSCs defined as $GFP^{+}sca\text{-}1^{+}c\text{-}kit^{+}$ cells in the spleen (n=4-6) and bone marrow (n=4) of BCR-ABL mice. Individual data points are shown from two independent experiments. Error bars represent mean ± SEM. *$p<0.05$.

The protective effect of selenium supplementation is reversed by inhibition of PPARγ

To further determine whether the protective effects of selenium were due to PPARγ activation in Se-S BCR-ABL mice, GW9662, a synthetic PPARγ antagonist, was used. General signs and symptoms of CML were increased in GW9662-treated Se-S BCR-ABL mice, when compared to vehicle treated Se-S BCR-ABL mice (Fig 3-2A-C). Prior to euthanizing BCR-ABL mice, blood analysis was used to monitor disease progression. These analyses indicated an increase in myeloid-derived leukocytes in the peripheral blood upon treatment with GW9662. Neutrophils were significantly increased and there was a trend of increased monocytes and eosinophils (Fig 3-1A-B). Total WBCs were twice as high in Se-S GW9662 BCR-ABL mice as compared to Se-S vehicle BCR-ABL mice (Fig 3-2A). Furthermore, flow cytometric analysis showed an increase in peripheral blood GFP in Se-S GW9662 mice (Fig 3-2B). Though the difference did not reach significance, there was over a two-fold increase in peripheral blood GFP when comparing the mean GFP expression in Se-S vehicle versus Se-S GW9662 BCR-ABL mice (Fig 3-2B). Splenomegaly was induced in Se-S GW9662 BCR-ABL mice, with over a two-fold increase in mass compared to the vehicle control group (Fig 3-2C).

Total disease burden was measured in the spleen and bone marrow by flow cytometric analysis of GFP. There was significantly higher number of GFP^+ cells in Se-S GW9662 BCR-ABL mice (Fig 3-2D). To further analyze the effect on the LSC population upon GW9662 treatment, GFP^+ cells from the spleen and bone marrow were analyzed for c-Kit and Sca-1 expression. $GFP^+Kit^+Sca\text{-}1^+$ (CML LSC-like) cells were significantly higher in both the spleen and bone marrow of Se-S GW9662 BCR-ABL mice when compared to Se-S BCR-ABL mice (Fig 3-3E). In the spleen, there was a two-fold increase in the number of CML LSCs. The bone marrow showed exactly a two-fold increase in CML LSCs (Fig 3-3E). Furthermore, functional CML LSCs in the spleen were analyzed by their ability to form colonies in methylcellulose media. Cells from GW9662 treated Se-S BCR-ABL mice formed significantly more LSC-CFUs

as compared to vehicle treated Se-S BCR-ABL mice (Fig 3-2F). As shown earlier with pioglitazone treatment, GW9662-treatment of BCR-ABL Se-S mice significantly decreased two prototypical PPARγ downstream genes, *Mrc1* and *CD68*, in splenocytes (Fig 3-2G). Taken together, these studies confirmed that PPARγ activation was essential for the protective effect in Se-S BCR-ABL mice.

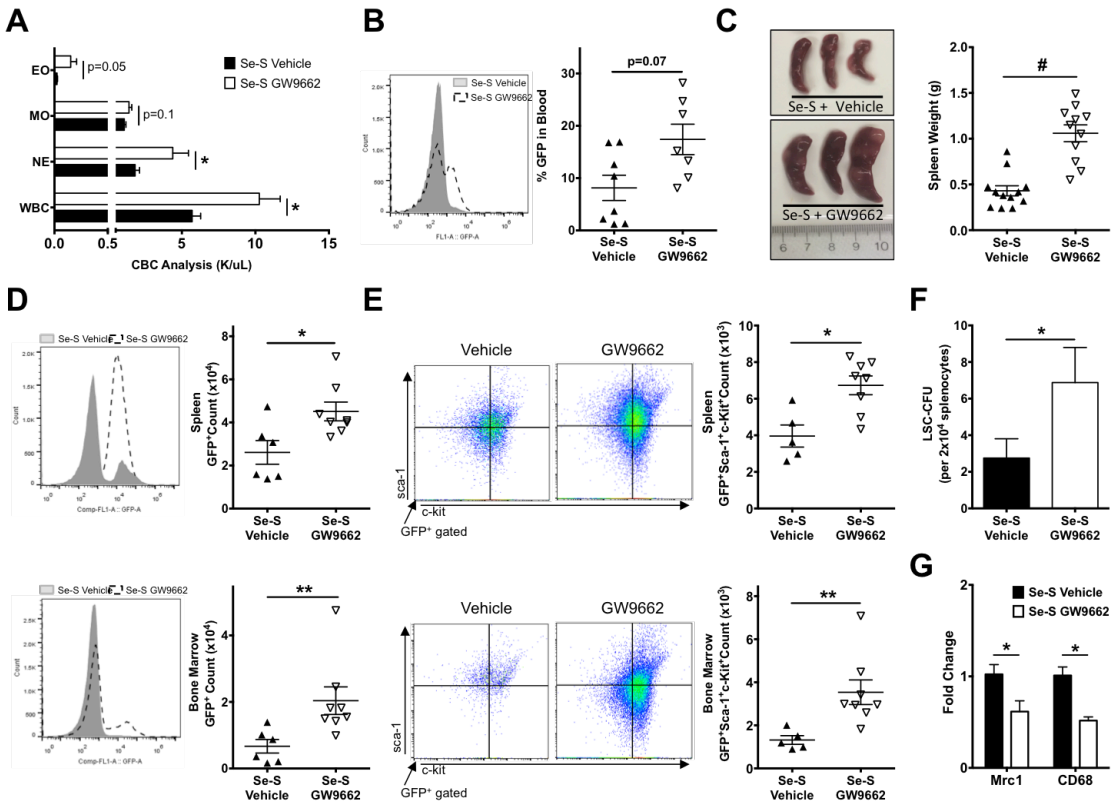

Figure **3-2**: GW9662 inhibits Se-S protection in CML mice.

Se-S mice, on diet for at least 8 weeks, were given daily *i.p.* injection of GW9662 (1mg/kg BW) starting one day prior to BCR-ABL transplant until euthanized. A) Complete blood count (CBC; K/µl blood; WBC (white blood cell); EO (eosinophils); MO (monocytes); NE, neutrophils) profile of Se-S BCR-ABL mice treated with or without GW9662. n=4. B) Flow cytometry of peripheral blood GFP expressed as percentage of gated input in Se-S BCR-ABL transplanted mice ($5x10^5$ events collected, gated on FSC). Representative histogram is shown along with individual data points. n=8. C) Representative image and spleen weight of Se-S BCR-ABL mice treated with or without GW9662 (n=11-12). D) Flow cytometric analysis of GFP in the spleen (top) and bone marrow (bottom) of Se-S BCR-ABL mice treated with or without GW9662. Representative histogram of GFP is shown on the left, with total data points on the right (n=6-8). Counts are shown. E) LSC analysis by flow cytometry in the spleen (top) and bone marrow (bottom) of Se-S BCR-ABL mice treated with or without GW9662. Cells were gated on GFP^+ population (shown in D). Scatter plots show representative example of GFP^+ gated cells on the sca-1 and c-kit axes. Total counts ($GFP^+sca\text{-}1^+c\text{-}kit^+$) are shown on right. Gates are based on GFP only and FMO controls. n=5-8. F) Total Se-S BCR-ABL splenocytes ($2x10^4$) were plated in technical triplicate in Methocult and counted on day 10. LSC-CFUs were counted and plotted as total counts. n=8. G) PPARγ inhibition with GW9662 in total Se-S BCR-ABL splenocytes by qPCR. n=4. Bars represent average of each biological mean ± SEM. Student two-way t-tests were preformed to compare groups. Error bars represent biological mean ± SEM. *p<0.05; **p<0.01; #p<0.0001.

GW9662 increases production and turnover of eicosanoids in Se-S BCR-ABL mice

Recently we have reported that the protective effect of selenium supplementation in BCR-ABL mice was mediated by production of endogenous CyPGs via the increased expression of H-PGDS and consequent production of Δ^{12}-PGJ_2 and 15d-PGJ_2 in Se-S BCR-ABL mice (23). Here we examined if the effect of increased disease burden in Se-S GW9662 mice was due to manipulation of the eicosanoid cascade. There was no difference in H-PGDS expression at both the RNA and protein level (data not shown). However, qPCR analysis in Lin^- BCR-ABL splenocytes revealed a significant increase in *15-Pgdh*, an enzyme responsible for the degradation of many prostaglandins, including Δ^{12}-PGJ_2 and 15d-PGJ_2 (Fig 3-3A). There was also a significant increase in *Txas1* and a trend of increased expression of *Ptges* (Fig 3-3A). However, at the protein level, the differences were more obvious and significant. 15-Pgdh, Ptges and Txas1 expression were all significantly higher in Se-S GW9662 BCR-ABL Lin^- splenocytes, with a two-fold increase in expression compared to Se-S control (Fig 3-3B). These data suggest inhibition of PPARγ with GW9662 in Se-S BCR-ABL mice shifts the production of eicosanoids: less endogenous PPARγ ligands are produced.

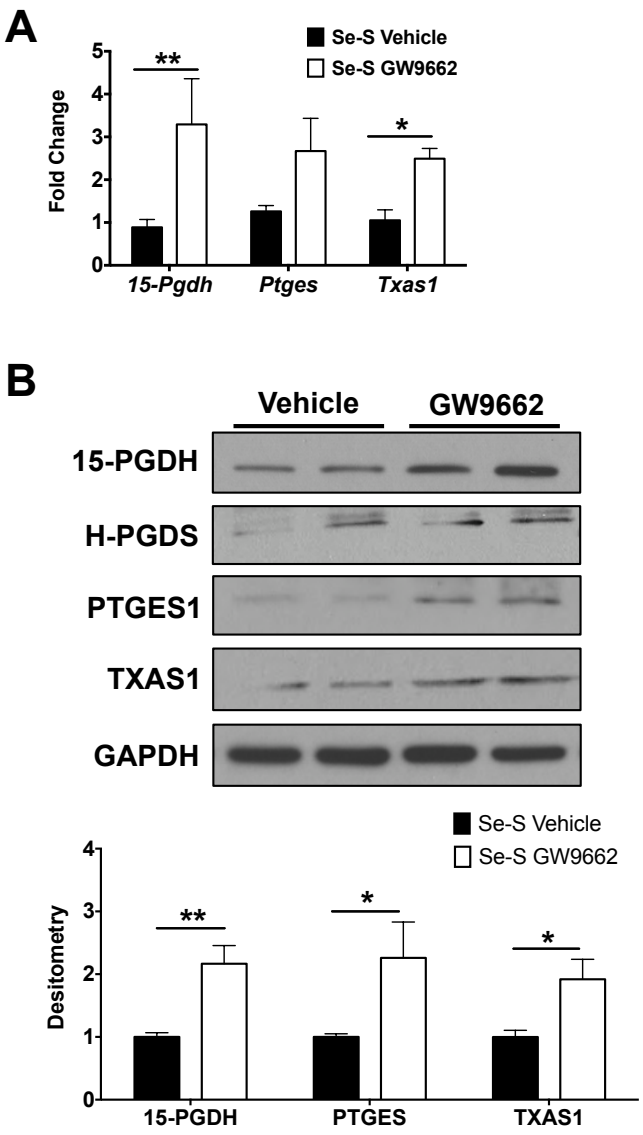

Figure **3-3**: Inhibition of PPARγ represses expression of Ptges-1 and Txas1 in Se-S CML progenitor cells.

Lineage negative (Lin^-) splenocytes from Se-S BCR-ABL mice treated with or without GW9662 were analyzed for RNA and protein expression. Se-S mice were given daily *i.p.* injection of GW9662 (1mg/kg BW) starting one day prior to BCR-ABL transplant until euthanized. n=4. A) qPCR expression as fold change compared to Se-S vehicle for each gene and normalized to 18s expression. B) Western blot analysis showing representative blot and densitometry (normalized to Se-S for each protein and relative to GAPDH). Bars represent biological mean ± SEM. All analyses were done in technical triplicate. *$p<0.05$; **$p<0.01$.

Δ^{12}-PGJ_2 and 15d-PGJ_2 concentrations are lower in Se-S GW9662 BCR-ABL mice

The observation of increased expression of 15-Pgdh was of particular interest due to its ability to degrade Δ^{12}-PGJ_2 and 15d-PGJ_2. Whole serum was collected from Se-S BCR-ABL mice and analyzed for concentration of these CyPGs by LC-MS/MS. Consistent with increased expression of 15-Pgdh, we observed a significant decrease in both Δ^{12}-PGJ_2 and 15d-PGJ_2 in the serum of Se-S GW9662 treated mice (Fig 3-4A). There was a 3.5-fold decrease in Δ^{12}-PGJ_2 and a three-fold decrease in 15d-PGJ_2 when mice were treated with GW9662 (Fig 3-4A). The upregulation of 15-Pgdh observed in Se-S GW9662 BCR-ABL mice (Fig 3-3) was accompanied by an increased concentration of the inactive CyPG 13,14-dihydro-15-keto-PGJ_2 (Fig 3-4A). Se-A pioglitazone treated BCR-ABL mice had a similar phenotype, in terms of CyPGs, as Se-S vehicle treated BCR-ABL mice. There was a significant increase in Δ^{12}-PGJ_2 and a trend of increased production of and 15d-PGJ_2 in Se-A pioglitazone treated BCR-ABL mice (Fig 3-4B). The breakdown product, 13,14-dihydro-15-keto-$PGDJ_2$, was decreased in Se-A pioglitazone BCR-ABL mice. These data implicate activation of PPARγ as being essential in the positive feedback loop of increased Δ^{12}-PGJ_2 and 15d-PGJ_2 production observed in Se-S mice. These data support our hypothesis that inhibition of PPARγ in Se-S mice increases CML disease burden due to decreased production of Δ^{12}-PGJ_2 and 15d-PGJ_2. The proposed involvement of selenium supplementation and PPARγ in CyPG production is shown in Fig 3-4C.

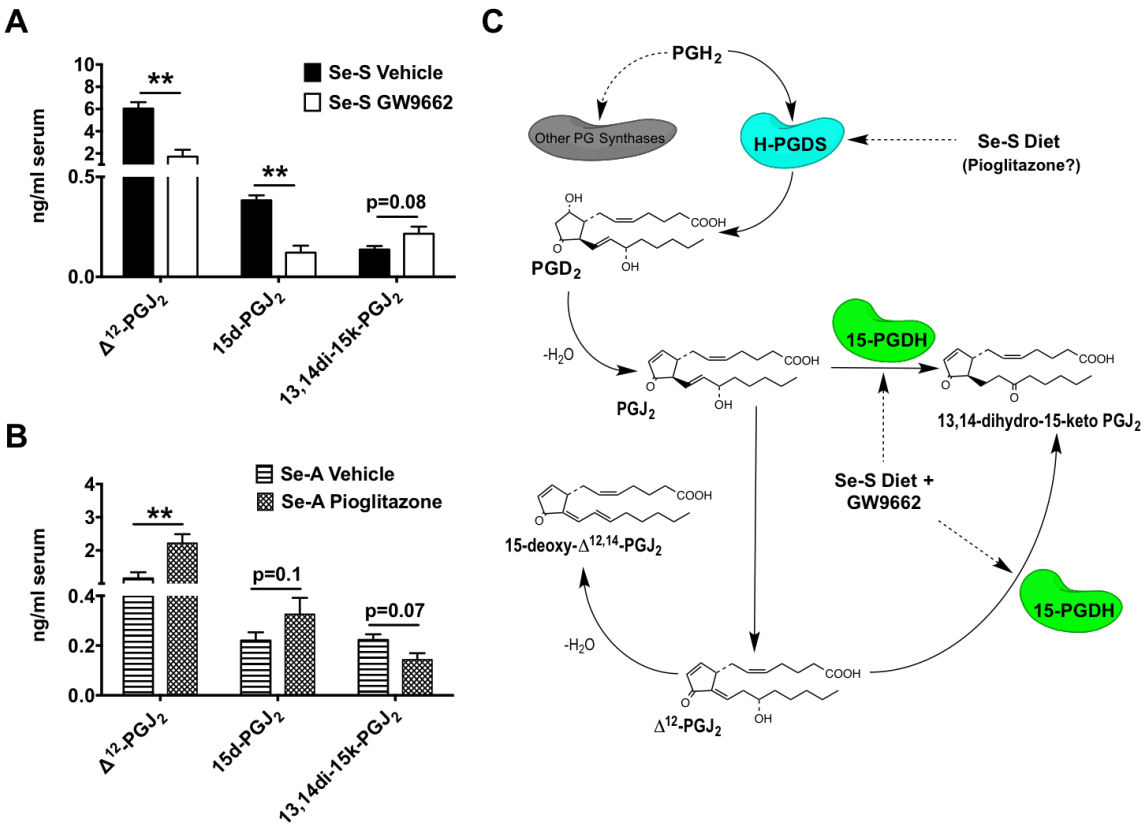

Figure **3-4**: Endogenous Δ^{12}-PGJ_2 and 15d-PGJ_2 concentrations are effected when PPARγ is targeted in CML mice.

Serum was subjected to C18 lipid extraction and LC-MS/MS MRM method for presence of Δ^{12}-PGJ_2, 15d-PGJ_2, and 13,14-dihydro-15-keto PGJ_2 (13,14di-15k-PGJ_2). A) Se-S BCR-ABL mice treated with or without GW9662. Se-S mice were given daily *i.p.* injection of GW9662 (1mg/kg BW) starting one day prior to BCR-ABL transplant until euthanized. n=3. B) Pioglitazone (5mg/kg BW) was given by *i.p.* injection daily starting one day prior to BCR-ABL transplant. n=5-6. Bars represent biological mean ± SEM. *$p<0.05$. C) Schematic of Se-S and GW9662 involvement in endogenous PPARγ ligand synthesis in BCR-ABL mice. Se-S diet increases H-PGDS, leading to subsequent increase in Δ^{12}-PGJ_2 and 15d-PGJ_2. Pioglitazone may also increase CyPGs. GW9662 in Se-S BCR-ABL mice increases 15-PGDH, which leads to breakdown of Δ^{12}-PGJ_2 and 15d-PGJ_2.

PPARγ inhibition leads to activation of STAT5a and LSC-maintaining genes

Recently pioglitazone was shown to decrease STAT5a activation in CML samples that were also treated with imatinib (16). We observed a similar effect in Se-S vehicle BCR-ABL mice when compared to Se-S GW9662 BCR-ABL mice. qPCR analysis of Lin^- splenocytes revealed a significant increase in *Stat5a, Cited2,* and *Hif2α* expression (Fig 3-5A). *Stat5a* was increased nearly 7-fold and *Hif2α* mRNA showed a 75-fold increase in Se-S GW9662 BCR-ABL samples. *Bcl2*, another STAT5 target and regulator of apoptosis (25), was slightly increased in GW9662 samples. The mean expression of *Cited2* was four fold higher in GW9662 samples. Being a master regulator of HSC quiescence, *Cited2* downstream targets, *Bmi1* and *Hes1*, also showed a trend of increased expression in GW9662 samples compared to Lin^- splenocytes from Se-S BCR-ABL mice (Fig 3-5A). At the protein level, the activation of Stat5a, in the form of phospho- (Y694)-Stat5a was seen in GW9662 samples (Fig 3-5B). Cited2 protein was significantly increased in Se-S GW9662 BCR-ABL samples. HIF2α protein was also increased upon GW9662 treatment in Se-S BCR-ABL cells. A similar pattern was seen in FACs sorted LSCs treated with or without selenium *ex vivo*. Selenium reduced expression of *Stat5a* and *Cited2* (Fig 3-5C). These studies confirm the essential role of selenium in inhibiting the activation of STAT5a and its downstream targets, through the activation of PPARγ.

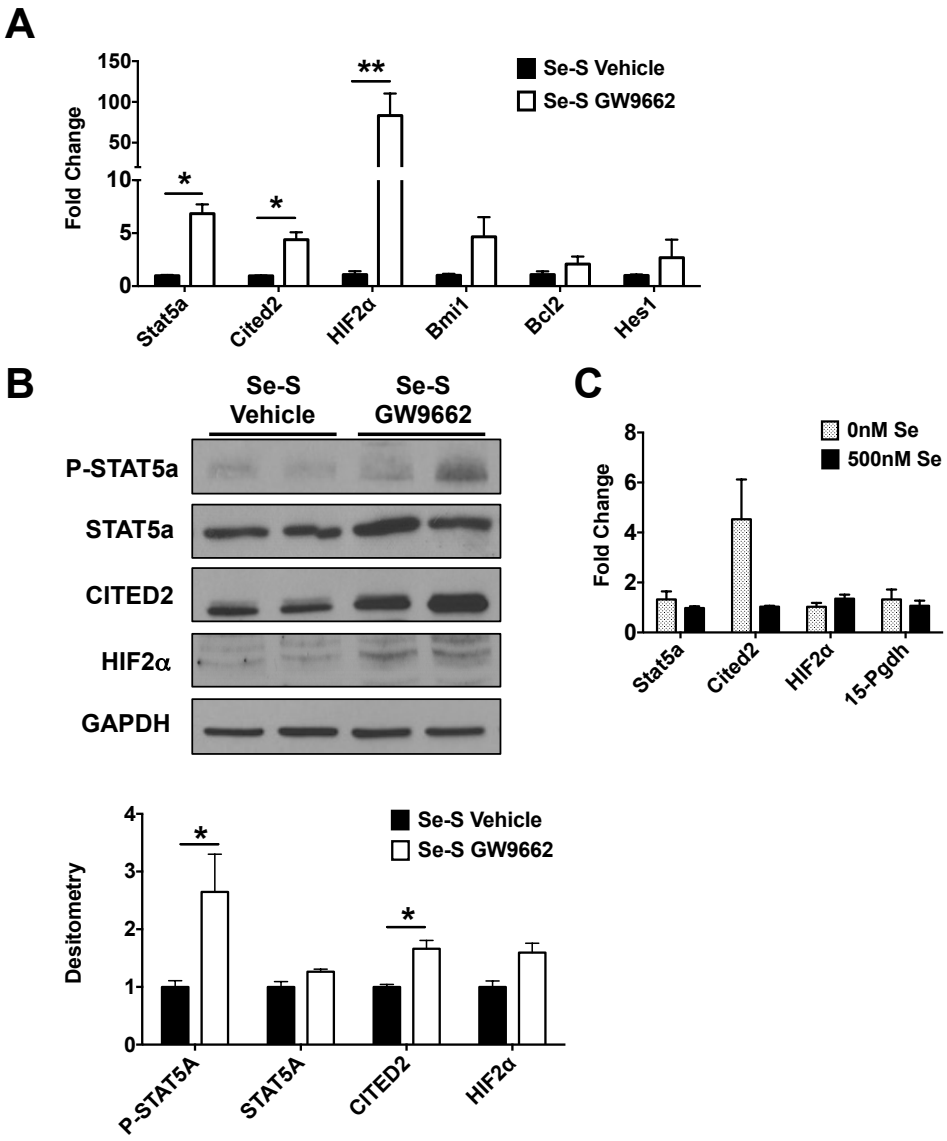

Figure **3-5**: PPARγ inhibition by GW9662 increases STAT5a and downstream targets.

A-B) Lineage negative (Lin^-) splenocytes from Se-S BCR-ABL mice treated with or without GW9662 were analyzed for RNA and protein expression of STAT5 and downstream targets. A) qPCR expression as fold change compared to Se-S vehicle for each gene and normalized to 18s expression. n=4. B) Western immunoblot analysis showing representative blot and densitometry (normalized to Se-S for each protein and relative to GAPDH). n=3-4. C) FACs sorted LSCs ($Lin^-GFP^+sca\text{-}1^+c\text{-}kit^+$) from Se-A BCR-ABL mice were treated for 6 hours with or without selenium (500 nM as selenite). qPCR analysis as fold change compared to 0nM treated cells for each gene and normalized to 18s expression. n=3. Bars represent biological mean ± SEM. All analysis was done in technical triplicate. *$p<0.05$; **$p<0.01$.

Discussion

The production of endogenous CyPGs, Δ^{12}-PGJ_2 and 15d-PGJ_2, serves as a key factor in the anti-leukemic effect of selenium supplementation in experimental CML (23). Use of NSAIDs, including indomethacin, and an inhibitor the H-PGDS, such as HQL-79, suggested that the cyclooxygenase-H-PGDS pathway of arachidonic acid metabolism was key to the endogenous production of CyPGs that eliminate LSCs. Most importantly, the causal relationship between cellular selenium levels and the activation of PPARγ was shown to affect a feed-forward mechanism leading to the enhanced production of CyPGs, while suppressing the other PGs significantly. This phenomenon was termed as the "eicosanoid class switching" that was effective under selenium-supplemented conditions (18). Here we demonstrate that such an activation of PPARγ serves as an essential component in the anti-leukemic effect of selenium supplementation. Use of either GW9662 in BCR-ABL Se-S mice or pioglitazone in Se-A mice further provide credence to the central role of PPARγ in the anti-leukemic activity of selenium at supra-nutritional levels.

Regulation of inflammation, differentiation, proliferation, and apoptosis by PPARγ makes this transcription factor an ideal candidate for CML therapy, as these pathways are central in leukemogenesis (16, 32). Our data demonstrating the antagonism of PPARγ in Se-S BCR-ABL mice, as well as the ability of pioglitazone to activate endogenous production of CyPGs, suggests that the anti-leukemic activity of selenium heavily depends on the activation of PPARγ. The activation of PPARγ in Se-S LSCs decreased the expression of Stat5a, which is known to occur through transcriptional repression to negatively affect the proliferation of quiescent LSCs (25).

Interestingly, inhibition of PPARγ with GW9662 treatment in Se-S BCR-ABL mice led

to the upregulation of Stat5a and its downstream targets, namely Cited2 and Hif2α compared to the LSCs from Se-S BCR-ABL mice. As mentioned earlier, CITED2 is a master regulator of HSC maintenance (33) and regulate LSCs in AML (34). HIFs regulate CITED2 expression (35). Given that Stat5a-driven expression of Cited2 and Hif2α are key to maintain quiescence of LSCs, the ability of selenium to significantly decrease the expression of Stat5a, Cited2, and Hif2α, which was abrogated upon treatment with GW9662 (in Se-S BCR-ABL mice), clearly supports our notion that selenium supplementation negatively affects the quiescence of LSCs to alleviate CML. In fact, these results corroborate well with the outcome of the serial transplantation experiments that were previously reported from our laboratory (23). Interestingly, conditional deletion of Cited2 is known to increase reactive oxygen species (ROS) in murine HSCs by decreasing glycolytic metabolism and increasing mitochondrial activity (36). These observations corroborate well with our previous and current findings where selenium supplementation increased ROS in LSCs (23) and this could be attributed to the inverse relationship between PPARγ activation and decrease in Cited2.

Surprisingly, upregulation of 15-Pgdh expression upon GW9662 treatment (Fig 3-3B) led to the increased oxidation of Δ^{12}-PGJ_2 and most likely impacted the bioactivity of this metabolite. Given the loss of unsaturation at carbon 13 (C13) and the oxidation of carbon 15 (15-OH), we believe that 13,14-dihydro-15-keto-PGJ_2 may not serve as a ligand for PPARγ. However, further research is needed to conclusively demonstrate if this metabolite functions as an endogenous ligand of PPARγ and if it impacts LSC quiescence. These studies ar currently underway and will be reported in the near future.

Clinically, it is debated whether the long-term use of pioglitazone may increase the risk of certain cancers, including bladder cancer (37). Pioglitazone is the only commonly available TZD due to other health risks associated with this class of drugs; rosiglitazone is highly regulated by the FDA due to associated increased myocardial infarction (38). The ability to activate PPARγ

with endogenous CyPGs through the manipulation of diet may be a promising alternative to TZD treatment in CML patients. Studies to determine the translatability of our data are needed. Additionally, it should be noted that excessive selenium supplementation might in itself come with health risks (39). However, the levels used in our studies are within the healthy range and should not cause additional risk.

In conclusion, we demonstrate the anti-leukemic effect of selenium as being mediated through activation of PPARγ by endogenous CyPGs (Figure 3.6). The reduction of the LSC population, and overall reduction in CML burden, was achieved without the addition of TKIs. These findings could be important in the treatment of CML. If dietary supplementation with selenium is sufficient for activation of PPARγ in CML LSCs, this would eliminate the off-target effects seen with synthetic PPARγ agonists. Further studies are needed to determine an optimal amount of selenium supplementation in conjunction with TKI therapy that effectively eliminates the CML LSC population and alleviate leukemogenesis.

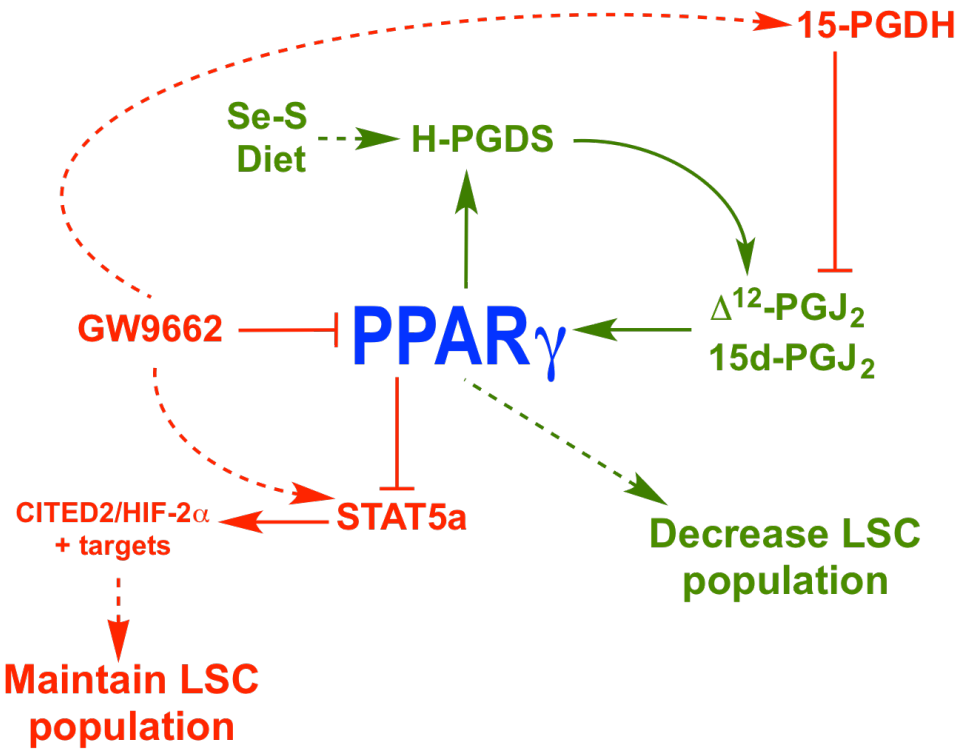

Figure **3-6**: PPARγ activation is central in the protective effect of Se-S in CML mice.

Se-S diet (green) increases expression of H-PGDS, leading to increased production of the PPARγ ligands Δ^{12}-PGJ_2 and 15d-PGJ_2. PPARγ activation feeds this cycle forward. GW9662 inhibition of PPARγ (red) is observed in two ways: first, it decreases STAT5a activation, and downstream transcription factors (including CITED2 and HIF2α) implicated in the maintenance of the LSC population; second GW9662 interrupts the positive feedback loop activated by selenium supplementation. Solid lines indicate a direct relationship; dashed lines indirect.

References

1. Goldman, J. M., and Melo, J. V. (2001) Targeting the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *The New England journal of medicine* **344**, 1084-1086
2. Apperley, J. F. (2015) Chronic myeloid leukaemia. *The Lancet* **385**, 1447-1459
3. Krause, D. S., and Van Etten, R. A. (2005) Tyrosine kinases as targets for cancer therapy. *The New England journal of medicine* **353**, 172-187
4. Hughes, T., Deininger, M., Hochhaus, A., Branford, S., Radich, J., Kaeda, J., Baccarani, M., Cortes, J., Cross, N. C., Druker, B. J., Gabert, J., Grimwade, D., Hehlmann, R., Kamel-Reid, S., Lipton, J. H., Longtine, J., Martinelli, G., Saglio, G., Soverini, S., Stock, W., and Goldman, J. M. (2006) Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood* **108**, 28-37
5. Gordon, M. Y., Dowding, C. R., Riley, G. P., Goldman, J. M., and Greaves, M. F. (1987) Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. *Nature* **328**, 342-344
6. Bedi, A., Zehnbauer, B. A., Barber, J. P., Sharkis, S. J., and Jones, R. J. (1994) Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. *Blood* **83**, 2038-2044
7. Hehlmann, R., Hochhaus, A., and Baccarani, M. (2007) Chronic myeloid leukaemia. *The Lancet* **370**, 342-350
8. Chu, S., McDonald, T., Lin, A., Chakraborty, S., and Huang, Q. (2011) Persistence of leukemia stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. *Blood* **118**, 5565-5570
9. Graham, S. M. (2002) Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* **99**, 319-325
10. Chomel, J.-C., Bonnet, M.-L., Sorel, N., Bertrand, A., Meunier, M.-C., Fichelson, S., Melkus, M., Bennaceur-Griscelli, A., Guilhot, F., and Turhan, A. G. (2011) Leukemic stem cell persistence in chronic myeloid leukemia patients with sustained undetectable molecular residual disease. *Blood* **118**, 3657-3660
11. Gerber, J. M., Qin, L., Kowalski, J., Smith, B. D., Griffin, C. A., Vala, M. S., Collector, M. I., Perkins, B., Zahurak, M., Matsui, W., Gocke, C. D., Sharkis, S. J., Levitsky, H. I., and Jones, R. J. (2011) Characterization of chronic myeloid leukemia stem cells. *American journal of hematology* **86**, 31-37
12. Nicholson, E., and Holyoake, T. (2009) The chronic myeloid leukemia stem cell. *Clinical Lymphoma and Myeloma* **9**, S376-381
13. Cilloni, D., and Saglio, G. (2012) Molecular pathways: BCR-ABL. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 930-937
14. Hamad, A., Sahli, Z., El Sabban, M., Mouteirik, M., and Nasr, R. (2013) Emerging therapeutic strategies for targeting chronic myeloid leukemia stem cells. *Stem cells international* **2013**, 724360
15. Holland, J. D., Klaus, A., Garratt, A. N., and Birchmeier, W. (2013) Wnt signaling in stem and cancer stem cells. *Current opinion in cell biology* **25**, 254-264
16. Prost, S., Relouzat, F., Spentchian, M., Ouzegdouh, Y., Saliba, J., Massonnet, G., Beressi, J.-P. P., Verhoeyen, E., Raggueneau, V., Maneglier, B., Castaigne, S.,

Chomienne, C., Chrétien, S., Rousselot, P., and Leboulch, P. (2015) Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature* **525**, 380-383

17. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83**, 803-812
18. Gandhi, U. H., Kaushal, N., Ravindra, K. C., Hegde, S., Nelson, S. M., Narayan, V., Vunta, H., Paulson, R. F., and Prabhu, K. S. (2011) Selenoprotein-dependent up-regulation of hematopoietic prostaglandin D2 synthase in macrophages is mediated through the activation of peroxisome proliferator-activated receptor (PPAR) gamma. *The Journal of biological chemistry* **286**, 27471-27482
19. Wang, D., and DuBois, R. N. (2006) Prostaglandins and cancer. *Gut* **55**, 115-122
20. Yousefi, B., Samadi, N., Baradaran, B., Shafiei-Irannejad, V., and Zarghami, N. (2016) Peroxisome proliferator-activated receptor ligands and their role in chronic myeloid leukemia: Therapeutic strategies. *Chemical biology & drug design* **88**, 17-25
21. Nelson, S. M., Lei, X., and Prabhu, K. S. (2011) Selenium levels affect the IL-4-induced expression of alternative activation markers in murine macrophages. *The Journal of nutrition* **141**, 1754-1761
22. Vunta, H., Belda, B. J., Arner, R. J., Channa Reddy, C., Vanden Heuvel, J. P., and Sandeep Prabhu, K. (2008) Selenium attenuates pro-inflammatory gene expression in macrophages. *Molecular nutrition & food research* **52**, 1316-1323
23. Gandhi, U. H., Kaushal, N., Hegde, S., Finch, E. R., Kudva, A. K., Kennett, M. J., Jordan, C. T., Paulson, R. F., and Prabhu, K. S. (2014) Selenium suppresses leukemia through the action of endogenous eicosanoids. *Cancer research* **74**, 3890-3901
24. Glodkowska-Mrowka, E., Manda-Handzlik, A., Stelmaszczyk-Emmel, A., Seferynska, I., Stoklosa, T., Przybylski, J., and Mrowka, P. (2016) PPARγ ligands increase antiluekemic activity of second- and thrid-generation tyrosine kinase inhibitors in chronic myeloid leukemia cells. *Blood cancer* **6**, e377
25. Hoelbl, A., Schuster, C., Kovacic, B., Zhu, B., Wickre, M., Hoelzl, M. A., Fajmann, S., Grebien, F., Warsch, W., Stengl, G., Hennighausen, L., Poli, V., Beug, H., Moriggl, R., and Sexl, V. (2010) Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO molecular medicine* **2**, 98-110
26. Finch, E. R., Kudva, A. K., Quickel, M. D., Goodfield, L. L., Kennett, M. J., Whelan, J., Paulson, R. F., and Prabhu, K. S. (2015) Chemopreventive Effects of Dietary Eicosapentaenoic Acid Supplementation in Experimental Myeloid Leukemia. *Cancer prevention research (Philadelphia, Pa.)* **8**, 989-999
27. Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L., and Baltimore, D. (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* **92**, 3780-3792
28. Hegde, S., Kaushal, N., Ravindra, K. C., Chiaro, C., Hafer, K. T., Gandhi, U. H., Thompson, J. T., van den Heuvel, J. P., Kennett, M. J., Hankey, P., Paulson, R. F., and Prabhu, K. S. (2011) Δ12-prostaglandin J3, an omega-3 fatty acid-derived metabolite, selectively ablates leukemia stem cells in mice. *Blood* **118**, 6909-6919
29. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)* **25**, 402-408
30. Pessina, A., Albella, B., Bueren, J., Brantom, P., Casati, S., Gribaldo, L., Croera, C., Gagliardi, G., Foti, P., Parchment, R., Parent-Massin, D., Sibiril, Y., and Van Den Heuvel, R. (2001) Prevalidation of a model for predicting acute neutropenia by colony

forming unit granulocyte/macrophage (CFU-GM) assay. *Toxicology in vitro : an international journal published in association with BIBRA* **15**, 729-740

31. Koschmieder, S., and Schemionek, M. (2011) Mouse models as tools to understand and study BCR-ABL1 diseases. *American journal of blood research* **1**, 65-75
32. Grommes, C., Landreth, G. E., and Heneka, M. T. (2004) Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists. *The Lancet. Oncology* **5**, 419-429
33. Kranc, K. R., Schepers, H., Rodrigues, N. P., Bamforth, S., Villadsen, E., Ferry, H., Bouriez-Jones, T., Sigvardsson, M., Bhattacharya, S., Jacobsen, S. E., and Enver, T. (2009) Cited2 is an essential regulator of adult hematopoietic stem cells. *Cell stem cell* **5**, 659-665
34. Korthuis, P. M., Berger, G., Bakker, B., Rozenveld-Geugien, M., Jaques, J., de Haan, G., Schuringa, J. J., Vellenga, E., and Schepers, H. (2015) CITED2-mediated human hematopoietic stem cell maintenance is critical for acute myeloid leukemia. *Leukemia* **29**, 625-635
35. Bakker, W. J., Harris, I. S., and Mak, T. W. (2007) FOXO3a is activated in response to hypoxic stress and inhibits HIF1-induced apoptosis via regulation of CITED2. *Molecular cell* **28**, 941-953
36. Du, J., Li, Q., Tang, F., Puchowitz, M. A., Fujioka, H., Dunwoodie, S. L., Danielpour, D., and Yang, Y.-C. C. (2014) Cited2 is required for the maintenance of glycolytic metabolism in adult hematopoietic stem cells. *Stem cells and development* **23**, 83-94
37. Lewis, J. D., Habel, L. A., Quesenberry, C. P., Strom, B. L., Peng, T., Hedderson, M. M., Ehrlich, S. F., Mamtani, R., Bilker, W., Vaughn, D. J., Nessel, L., Van Den Eeden, S. K., and Ferrara, A. (2015) Pioglitazone Use and Risk of Bladder Cancer and Other Common Cancers in Persons With Diabetes. *JAMA* **314**, 265-277
38. Woodcock, J., Sharfstein, J. M., and Hamburg, M. (2010) Regulatory action on rosiglitazone by the U.S. Food and Drug Administration. *The New England journal of medicine* **363**, 1489-1491
39. Ogawa-Wong, A. N., Berry, M. J., and Seale, L. A. (2016) Selenium and Metabolic Disorders: An Emphasis on Type 2 Diabetes Risk. *Nutrients* **8**, 80

Chapter 4

Activation of the DP receptor by cyclopentenone prostaglandins decrease myeloid leukemia

Abstract

The mechanism through which the cyclopentenone prostaglandins (CyPGs), Δ^{12}-PGJ_2 and Δ^{12}-PGJ_3, meditate their anti-leukemic effect is not fully understood. Endogenous production of these CyPGs through dietary manipulation leads to elimination of the chemoresistant leukemia stem cell (LSC) population. CyPGs can act through binding to a specific family of G protein coupled receptors (GCPR), DP1 and CRTH2, collectively known as DP receptors. Here we investigate whether the anti-leukemic effect of CyPGs is mediated through binding to DP receptors on LSCs in three experimental models of leukemia, which include two models of CML (BCR-ABL and the TKI-resistant T315I BCR-ABL) and one model of AML (MLL-AF9). The LSC-progenitor population was decreased; both *in vitro*, decreased LSC-colony forming units, and *in vivo,* when DP receptors were activated with CyPGs and other synthetic receptor agonists. Blocking of the DP receptors *in vitro* with synthetic antagonists prevented the anti-leukemic effect. Taken together, our results implicate DP receptors as a novel target in the treatment of leukemia.

Introduction

Current chemotherapies for myeloid leukemias do not eliminate the cancer causing leukemia stem cell (LSC) population. Developing therapies that eliminate LSCs is the holy grail of cancer therapies, as it could lead to curing the disease. We have shown an anti-leukemic effect of certain cyclopentenone prostaglandins (CyPGs), namely Δ^{12}-PGJ_2 and Δ^{12}-PGJ_3 (1-3). These CyPGs specifically reduce the LSC population. Endogenous CyPGs cause changes in redox homeostasis by exacerbated oxidative stress in LSCs leading to the activation of the tumor suppressor p53 (2), though the mechanism is not fully understood. There are two distinct mechanisms though which CyPGs act: 1) binding to prostanoid-specific G protein-coupled receptor (GPCR) (4) and/or 2) covalently modifying select cysteine residues in various intracellular proteins, including peroxisome proliferator activated receptor gamma (PPARγ) (5-7).

Endogenous prostanoids, which collectively include prostaglandins (PGs), thromboxanes, and prostacyclins, are autacoids, acting as localized hormones in or near the site where they are produced (8). On effector cells, prostanoids bind to a specific seven-transmembrane GPCRs. The prostanoid receptors are named based on the prostanoid that binds with highest affinity (9), or for which binding was first confirmed. Two GPCRs have been identified to specifically bind PGDS-derived CyPGs (prostaglandin D synthases- lipocalin-type or hematopoietic) with high affinity: DP/DP1 (PTGDR), herein referred to as DP1, and CRTH2 (chemoattractant receptor-homologous molecule expressed on T-helper 2 cells; GPR44; DP2). Collectively, these GPCRs are referred to as DP receptors.

Though the DP1 receptor is expressed in most tissues at low levels (10), activation is mostly studied in the context of allergic response (11). In the immune system, DP1 was first

implicated for binding PGD_2 released from IgE-activated mast cells (12). DP1 activation leads to G_s-mediated increases in intracellular cAMP. This leads to increased protein kinase A (PKA) and changes in gene regulation (10, 13). CRTH2 was first identified on T-helper 2 cells for its ability to bind PGD_2 during an allergic response (14). This receptor is distinct from most prostanoid receptors; it is evolutionarily more similar to other chemoattractant receptors and it shares highest amino acid sequence homology with members of the leukocyte chemoattractant receptor superfamily, which includes FMLP receptor, C3a receptor and C5a receptor (14). The CRTH2 receptor couples to a G_i-type protein (15), upon ligand binding causing inhibition of cAMP, activation of PLCβ (phospholipase C beta) that increases mobilization of intracellular Ca^{2+} mediated by IP3 (inositol triphosphate) and DAG (diacylglycerol). Increased intracellular Ca^{2+} then activates protein kinase C (PKC).

Though mostly implicated for their role in allergies and asthma, DP receptor activation is implicated in diverse physiological responses. For example, PGD_2 binding to DP1 in the brain regulates sleep (16, 17) and in platelets prevents aggregation (18). It was originally thought that each prostanoid carried out its physiological activity through a unique GPCR (19). However, it is now known that multiple ligands can bind to the same receptor, but a particular ligand can cause a different cellular response when bound to the same receptor (20). For example, ZK118182, an agonist of both DP1 and CRTH2 receptors, increases histamine production in mast cells and airway resistance. Interestingly, though Δ^{12}-PGJ_3 binds to mouse DP1 with reasonable affinity and CRTH2 with high affinity (unpublished data), it does not cause degranulation of bone-marrow derived mast cells nor cause any increase in airway hyper-responses in mice (21). Of interest to us is to understand the role of the DP receptors in the treatment of cancer.

Synthetic antagonists and agonists for DP1 and CRTH2 have been synthesized to study the biological activity of these receptors. Antagonists for DP receptors have been developed to treat asthma (18), but can also be utilized to define the role of these receptors in cancer research.

One such antagonist, Cay10471, is a highly selective antagonist for CRTH2. Cay10471 blocks PGD_2-mediated chemotaxis, eosinophil release from the bone marrow, as well as respiratory burst (22). Many J family CyPGs have been identified as potent DP1 and CRTH2 agonists in both mice and humans, though CRTH2 binding is favored. Δ^{12}-PGJ_2 can bind to human DP1 and CRTH2, with concentrations in the physiological range: Ki ~100 nM and ~7 nM for DP1 and CRTH2, respectively (23). In mice, Δ^{12}-PGJ_2 binding to CRTH2: ~ Ki 400 nM (4). 15d-PGJ_2 binds to both human (23) and mouse CRTH2 several orders of magnitude higher than binding to PPARγ (24). PGD_2, PGJ_2, and the synthetic agonist BW245C, bind with highest affinity to DP1 (25). In addition to Δ^{12}-PGJ_2 and 15d-PGJ_2, the PGD_2 metabolite 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2) bind with highest affinity to CRTH2 (15). Studies conducted in from our laboratory suggest Δ^{12}-PGJ_3 can bind to both DP1 and CRTH2, with a higher affinity towards the latter (unpublished results).

Both DP1 and CRTH2 are expressed in various cancers. DP1 and CRTH2 receptors are expressed by the human colon cancer cell line HT-29 (26). In $Apc^{Min/+}$ mice, which are susceptible to intestinal adenoma formation, Dp1 knockout increased total number of tumors (27). In a screen of 277 primary gastric tumors, DP-positive (DP1 or CRTH2) was associated with lymph node metastasis, lymphatic invasion, and venous invasion. (28). While the receptors appear to be important, little has been done to follow-up on the clinical implication in cancer management.

Here, we investigate the role of the DP1 and CRTH2 receptors in models of myeloid leukemias. Three experimental models of leukemia were used: two models of CML (BCR-ABL and the TKI-resistant T315I BCR-ABL) and one model of AML (MLL-AF9). Particular interest was in Δ^{12}-PGJ_3, however a panel of natural and synthetic DP receptor agonists was studied for the ability to decrease LSC burden, in all models of leukemia. Blocking of DP receptors with

synthetic antagonist prevented the anti-leukemic effect of CyPGs. This research demonstrates a unique and important role for prostanoid receptors in cancer treatment.

Materials and Methods

Animals

Three-week-old C57BL/6 mice (Taconic Biosciences, Hudson, NY) were housed three to four mice per cage in a temperature- and humidity-controlled room with a 12-hour light-dark cycle. All mice were 11-16 weeks of age at sacrifice, unless noted otherwise. Mice over 40 grams at time of transplant were not used. The Institutional Animal Use and Care Committee (IACUC) at The Pennsylvania State University preapproved all procedures.

Generation of experimental myeloid leukemias

Mouse models of myeloid leukemias were generated by transducing bone marrow stem and progenitor cells with retrovirus carrying BCR-ABL (CML), BCR-ABL T315I (TKI resistant CML), or MLL-AF9 (AML) and transplanting them into sub-lethally irradiated (475 rads) mice. Isolation and transduction of HSCs was performed as described earlier (1, 29). In all experimental models, transduced bone marrow cells were passaged through mice at least two times prior to transplantation into experimental mice. Disease progression was monitored by complete blood count (CBC) analysis, evaluating peripheral blood for leukocytosis on a Hemavet 950FS equipped with a veterinary software program. All mice were sacrificed on day 21 post-transplant. Spleen and bone marrow were isolated and used for characterization as described below. Any deviation from this transplantation system is described in figure legends.

Flow cytometry of CML LSCs

Whole spleen and bone marrow were collected and red blood cells (RBCs) were lysed with ACK lysis buffer (155mM NH_4Cl, 12mM $KHCO_3$, 0.1mM EDTA). Lineage negative cells were isolated following manufacturer's protocol (Stem Cell Technologies, Vancouver, British Columbia). Cells were stained for Ly-6A/E (sca-1) and CD117 (c-kit) (BD Biosciences, San Jose,

CA). Lineage negative white blood cells (WBCs) and the presence of LSCs were analyzed on Accuri C6 or Fortessa LSR flow cytometers (BD Biosciences, San Jose, CA). KSL (c-kit$^+$sca-1$^+$Lin$^-$) LSCs were defined as Lin$^-$ selected cells that stained positive for c-kit, sca-1, and GFP (present in BCR-ABL plasmid).

LSC-Colony Forming Units (LSC-CFU)

Total splenocytes or bone marrow from leukemic mice was plated in M3231 Methocult (Stem Cell Technologies, Vancouver, British Columbia). Media was supplemented with IL-3 (2.5ng/ml; R&D Systems, Minneapolis, MN), stem cell factor (SCF; 50ng/ml; Gold Biotechnology, St. Louis, MO), growth differentiation factor 15 (GDF-15; 30ng/ml; Biomatik, Wilmington, DE), and sonic hedgehog (Shh; 25ng/ml; Gold Biotechnology, St. Louis, MO). On days 7-10, colonies were counted. LSC-colony forming units (LSC-CFU) were defined based on their size (large: >100 cells at high magnification), shape (perfectly circular), density (dark brown/black color), and edge (clearly defined edge with limited differentiation). Any variation from LSC-CFU formation was also identified: in some cases granulocyte-macrophage progenitor colony forming units (CFU-GM) were observed (30). Each biological sample was plated in triplicate. The average number of LSC colonies from each biological sample was tabulated for analysis.

Basophil Activation

Human PBMCs (All Cells, Alameda, CA), from individuals taking no drugs, including NSAIDs/dietary supplements, for >1 week, were analyzed using the Flow Cast Basophil Activation Test (Bühlmann Laboratories, Schönenbuch, Switzerland). Two positive controls were included in the protocol (positive control and fMLP positive control). DP receptor agonists were incubated for 30 minutes prior to analysis. DP receptor antagonists were added 10 minutes prior to addition of agonists. Basophils were identified as CCR3$^+$ SSC^{low}; activated basophils CD63$^+$.

DP receptor agonists and antagonists

All synthetic receptor agonists were purchased from Cayman Chemicals (Ann Arbor, MI) with the exception of Δ^{12}-PGJ_3 (which was synthesized in the laboratory). The specific target receptor is shown in Figure 4-1. Ethyl acetate stocks of all agonists/antagonists were stored at -80 °C in an airtight glass vial. For *in vitro* experiments, compounds were blown down with nitrogen gas and re-suspended in PBS (with <0.01 % DMSO). *In vivo*, treatments were reconstituted daily. Compounds were re-suspended in PBS (<0.01 % DMSO) and administered (0.05 mg/kg body weight) by *i.p.* injection (500 µl volume). Treatments began on day seven post-transplant.

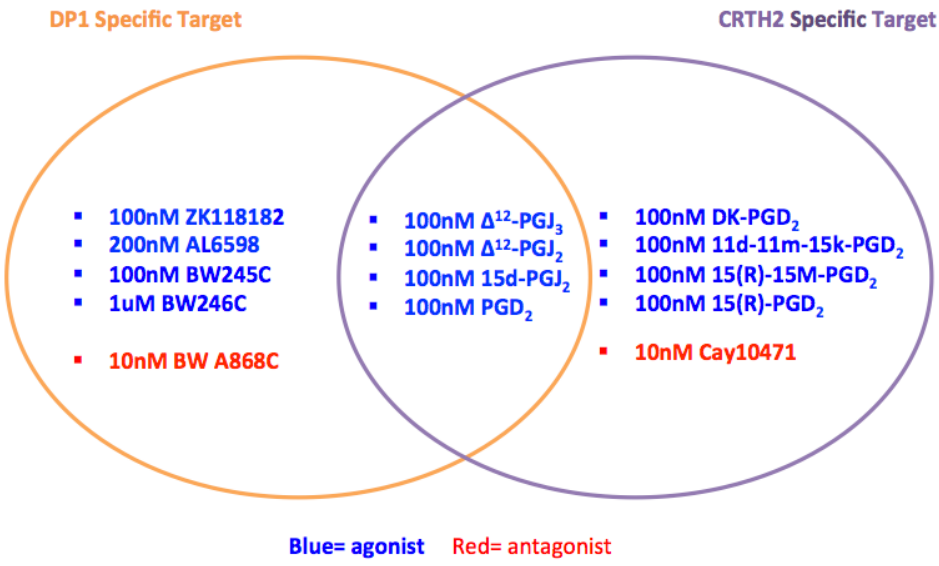

Figure **4-1**: DP receptor agonists and antagonists.

Agonists and antagonists specific for DP1 or CRTH2 receptor are listed, with *in vitro* concentrations. D and J family prostaglandins in the middle have dual activity. Abbreviations: 11-deoxy-11-methylene-15-keto-PGD_2 (11d-11m-15k-PGD_2); 15(R)-15-methyl PGD_2 (15(R)-15M-PGD_2).

shRNA knockdown of CRTH2 in BCR-ABL CML

Bacterial stocks containing a plasmid shRNA clone targeting Crth2 (gpr44) were purchased (Transomic, Huntsville, AL). Bacterial stocks were plated on ampicillin (100 mg/ml) treated agar plates. Single colonies were selected after ~18 hours of growth in humidified 37 °C chamber, and added to an Luria Broth (LB) starter culture: 3 ml LB with ampicillin (100 mg/ml). After ~6-8 hours shaking incubation (>200 rpm) at 37 °C, 150 µl of starter culture was added to large-scale culture: 150 ml LB with ampicillin (150 mg/ml). Plasmid DNA was isolated using Qiagen Plasmid MaxiPrep (Qiagen, Hilden, Germany), following standard protocol. Plasmid DNA was quantified for supercoiled concentration by electrophoresis and stored in nuclease-free water at -20 °C. All plating and culturing of bacteria was conducted with "flame sterilization." LB was autoclaved and stored at room temperature; ampicillin was sterile filtered, stored at 4 °C, and added to LB at time of adding bacteria.

Retroviral particles were generated by transducing HEK 293T cells with a "complex" of plasmid DNA with Mirus TransIT 293 transfection reagent (Mirus, Madison, WI), following manufactories instructions. Retroviral stocks were filtered (0.45 µm polyethersulfone sterile filter) and stored at -80 °C. No concentration of retrovirus was conducted.

Whole bone marrow from BCR-ABL mice (RBCs lysed and rinsed) was transduced with retrovirus (~4ml/ two mice- four femurs total) for 3-5 hours. Cells were rinsed, strained through 70 µm filter, and re-suspended in conditioned IMDM (previously described) for 4-12 hours. Trypan blue counting was conducted prior to transplant.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Unless noted, nonparametric tests, including one-way ANOVA and un-paired two-tailed student t-test, were used where appropriate. The vehicle group (DMSO or PBS) was used as the control, unless otherwise noted, for t-tests. For ANOVA, all groups were compared,

unless noted. Variation from these analyses is described in figure legends. All experiments were run in technical duplicate or triplicate, as described. Bars represent biological mean ± SEM: *$p<0.05$, **$p<0.01$, ***$p<0.001$, #$p<0.0001$.

Results

DP receptors are expressed in experimental leukemia cells

The expression of DP receptors on leukemic cells has not been reported. In order to determine if the CyPGs of interest, namely Δ^{12}-PGJ_2 and Δ^{12}-PGJ_3, can act through these receptors, first we determined the expression of Dp1 and Crth2 in our three models of leukemia (BCR-ABL, T315I, and MLL-AF9). Mice were transplanted with leukemic bone marrow expressing BCR-ABL, T315I, or MLL-AF9 and the disease was allowed to progress. Splenocytes were isolated from leukemic mice. These cells are highly enriched for leukemic cells. In the case of BCR-ABL Lin^- selection was conducted. Protein expression showed expression of both Dp1 and Crth2 in all models of leukemia (Fig 4-2A). The enrichment of this receptor was seen in the sorted BCR-ABL CML-LSC population over both Lin^- or whole splenocytes, at both the protein (Fig. 4-2B) and RNA (Fig. 4-2C) level.

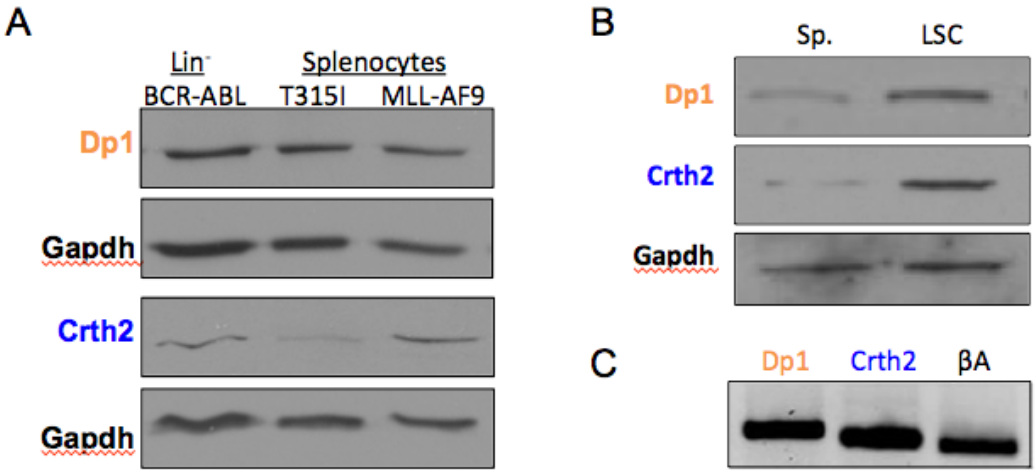

Figure **4-2**: The DP receptor is expressed in leukemia models.

A) Protein expression from Lin- (BCR-ABL) or whole splenocytes (T315I and MLL-AF9) in leukemic mice. Gapdh was used as a loading control. B) Protein expression from whole spleen (Sp.) or FACS sorted splenic LSCs in BCR-ABL mice. Gapdh was used as a loading control. This is a representative n=1 of 3 independent experiments. C) BCR-ABL LSCs were FACS sorted for $GFP^+c\text{-}kit^+sca\text{-}1^+$ from the spleen of leukemic mice. Dp1 and Crth2 gene expression is shown by semi-quantitative PCR. Beta-actin (βA) served as an experimental control.

CyPGs do not effect activation of human-basophils

The use of Δ^{12}-PGJ_2 or Δ^{12}-PGJ_3 for treatment of human leukemias requires that the therapy have no obvious detrimental effects. As granulocyte activation with PGD_2 via DP1 and CRTH2 is implicated in allergies, we tested a panel of natural and synthetic DP receptor agonists on human basophil activation (Fig 4-3A). The concentrations used were extrapolated based on known Ki affinities, and were the levels determined effective for use in leukemia models. We focused our safety studies on basophils, as they express both the DP1 and CRTH2 receptors (31). There was no significant activation of basophils in any sample, from both male and female individuals (Fig 4-3A). Since the primary interest was in Δ^{12}-PGJ_2 and Δ^{12}-PGJ_3, these CyPGs were administered up to 1 μM. Even up to this high concentration, which is over 10-fold the IC_{50} (25), there was no detectable activation of basophils (Fig 4-3B,C). Given that both receptors are expressed in these cells, in a subset of experiments, the CRTH2 receptor was blocked with the specific antagonist Cay10471 prior to addition of Δ^{12}-PGJ_2. The antagonist, even at 100 nM, ~100-fold excess of the reported IC_{50} for CRTH2 (32), did not affect basophil activation (Fig 4-3C). Taken together, these data suggest no detrimental effects of the doses of CyPGs used in leukemia experiments. These data further corroborate the lack of airway hyper-responses in mice treated with CyPG, along with parasympathomimetic drug, methacholine (21).

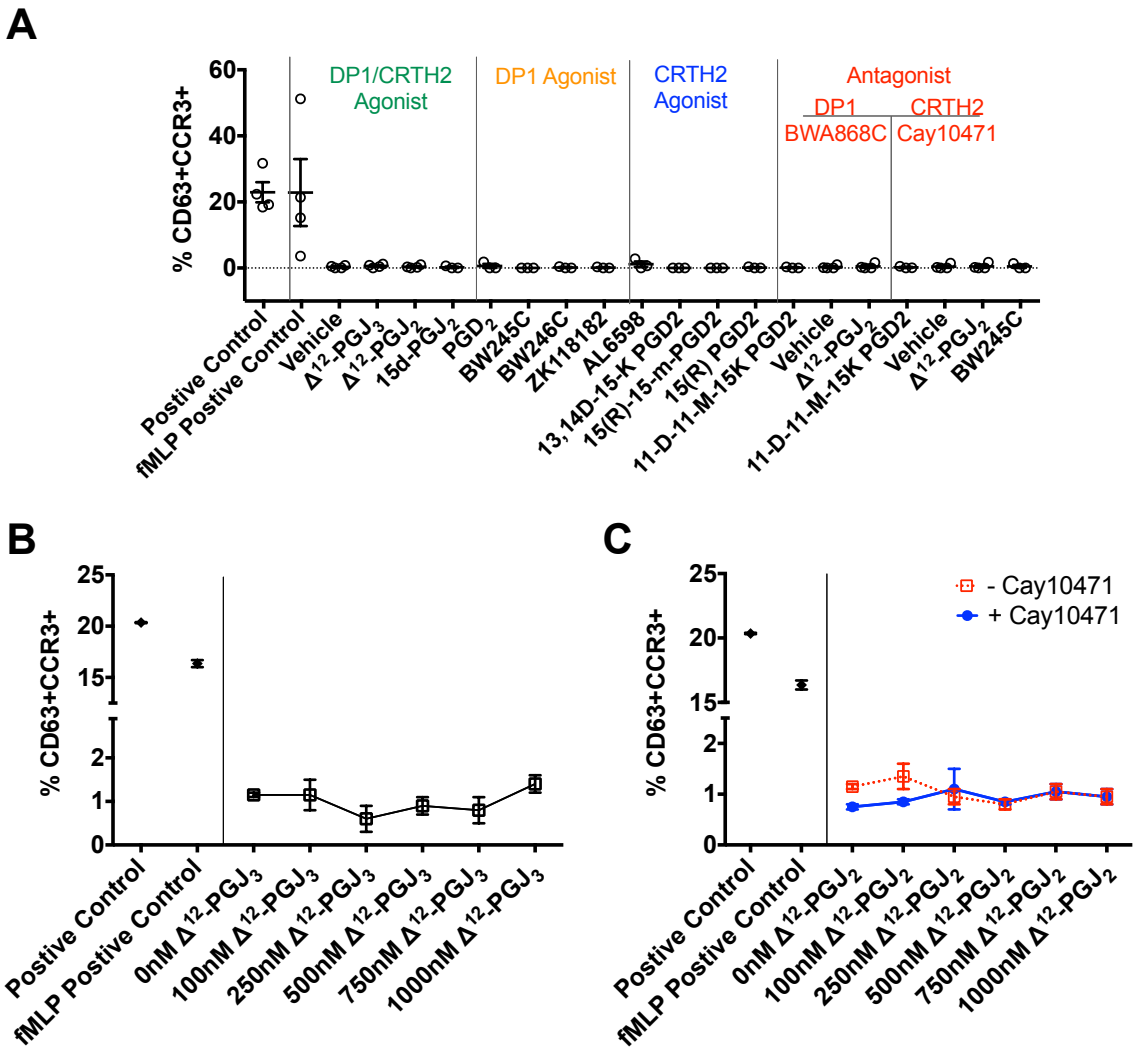

Figure **4-3**: Treatment with DP receptor agonists does not cause excess activation of human basophils.

Human PBMCs were treated with activation controls provided or with DP receptor antagonists and/or agonists. Where appropriate, antagonists were added 10 minutes prior to addition of agonist. Standards and agonists were incubated for 30 minutes. A) DP receptor panel. Cay10471 and BWA868C were added at 10nM. All agonist treatments were performed at 100 nM, with the exception of AL6598 (200 nM) and BW246c (1 μM). Basophils were identified as $CCR3^{+}$ $SSC^{low.}$ Activated basophils $CD63^{+}$. N=3-4. All DP receptor agonists caused significantly lower basophil activation ($p<0.05$) as compared to either positive control or fMLP positive control by one-way ANOVA. B) Dose response of Δ^{12}-PGJ_3 (0-1000 nM) on basophil activation n=1 in duplicate. C) Dose response of Δ^{12}-PGJ_2 (0-1000 nM) with and without CRTH2 antagonist Cay10471 (Cay10471 dose at 100nM). n=1 in duplicate.

Activation of the DP receptor limits LSC colony formation in myeloid leukemias

To determine the effect of DP receptor activation in LSCs, colony assays designed to enrich progenitor cells were conducted in three models of myeloid leukemia. Only units defined as LSC-CFUs were counted and reported values are relative to control for each biological replicate.

MLL-AF9 generated AML presents with a homogenous population of stem-like leukemic cells in the spleen of mice, after migration from the bone marrow. With either DP1 or CRTH2 activation, AML LSC-CFU counts were significantly decreased by nearly 50%, as compared to vehicle control (Fig 4-4A). Interestingly, all agonists displayed similar ability to limit the AML LSC-CFUs. There was no preference to one agonist and similar findings were seen across all individual experiments. When AML cells were pre-treated with BWA868C, a Dp1 antagonist, followed by Δ^{12}-PGJ_2 treatment, the formation of AML-LSC-CFUs was not different from control. This indicates that the activity of Δ^{12}-PGJ_2 was mediated through their ability to activate the Dp1 receptor.

CML progresses differently than AML and the population of cells in the spleen or bone marrow of mice is more heterogeneous. Whereas AML is quick and aggressive, with mice succumbing to disease within a window of two weeks, CML can take longer to process. For preliminary screening of DP agonists, whole BCR-ABL splenocytes were used in a similar assay to AML, with diverse DP1 and CRTH2 agonist treatments. Interestingly, in contrast to AML, unsorted BCR-ABL LSC-CFUs varied with different agonist treatment. Where the dual active PGs (Δ^{12}-PGJ_3, Δ^{12}-PGJ_2, 15d-PGJ_2, PGD_2) and the majority of other agonists showed a significant 50% reduction in LSC-CFUs, the DP1 specific agonist BW245C decreased BCR-ABL LSC-CFUs by nearly 10-times (10 % the LSC-CFUs as control) (Fig 4-4B). Interestingly, the CRTH2 agonist 15(R)-PGD_2 did not change BCR-ABL LSC-CFUs from control.

FACS sorted BCR-ABL LSCs ($Lin^{-}c\text{-}kit^{+}sca\text{-}1^{+}GFP^{+}$) cells were plated in the same way. In this case, the pattern of sorted BCR-ABL LSC-CFU formation was varied. Though statistical analysis was not possible (n=2), the trends can be interpreted. Δ^{12}-PGJ_3 and Δ^{12}-PGJ_2 reduced sorted BCR-ABL LSC-CFUs by nearly 80 %, as compared to vehicle control (Fig 4-4C). 15d-PGJ_2 and PGD_2 cased a 40 % reduction in BCR-ABL LSC-CFUs. DP1 specific agonists had variable ability to decrease sorted BCR-ABL LSC-CFUs: ZK118182 (70 % reduction); AL6598 (60 % reduction); BW245C (40% reduction); BW246C (20% reduction). The CRTH2 agonists (DK-PGD_2, 15(R)-15M-PGD_2, 15(R)-PGD_2) reduced sorted BCR-ABL LSC-CFUs to 20 % what was observed in the control (80 % reduction). However, the highly specific CRTH2 agonist, 11-deoxy-11-methylene-15-keto PGD_2 (11d-11m-15k-PGD_2) only decreased sorted BCR-ABL LSC-CFUs by 30% as compared to control. This was unexpected. A subset of cells was pretreated, with the DP1 antagonist BWA868C or the CRTH2 antagonist CAY10471, prior to treatment with Δ^{12}-PGJ_2. Pre-treated cells showed increased BCR-ABL LSC-CFUs compared to Δ^{12}-PGJ_2 treatment alone (Δ^{12}-PGJ_2: 20 % of control; BWA868C + Δ^{12}-PGJ_2: 30 % of control; CAY10471 + Δ^{12}-PGJ_2: 85 % of control). These data suggest that Δ^{12}-PGJ_2 primarily acts through Crth2 to elicit the anti-leukemic effect on sorted BCR-ABL LSC-CFU formation (Fig 4-4C).

In a subset of experiments with T315I BCR-ABL splenocytes, all DP receptor agonists reduced T315I LSC-CFUs (Fig 4-4D). Δ^{12}-PGJ_3 reduced T315I LSC-CFUs by 50%. The most drastic decreases were seen with CRTH2 agonist treatments, however these data need to be replicated in order to make any conclusions. In nearly all cases, across leukemia models, DP1 and CRTH2 activation decreased the formation of LSC-CFUs. In Figure 4-4E, LSC-CFU examples are shown. It appears that both DP receptors are important in the anti-leukemic function of PGs. However, CRTH2, in most cases, mediated the most drastic reduction in LSC-CFUs.

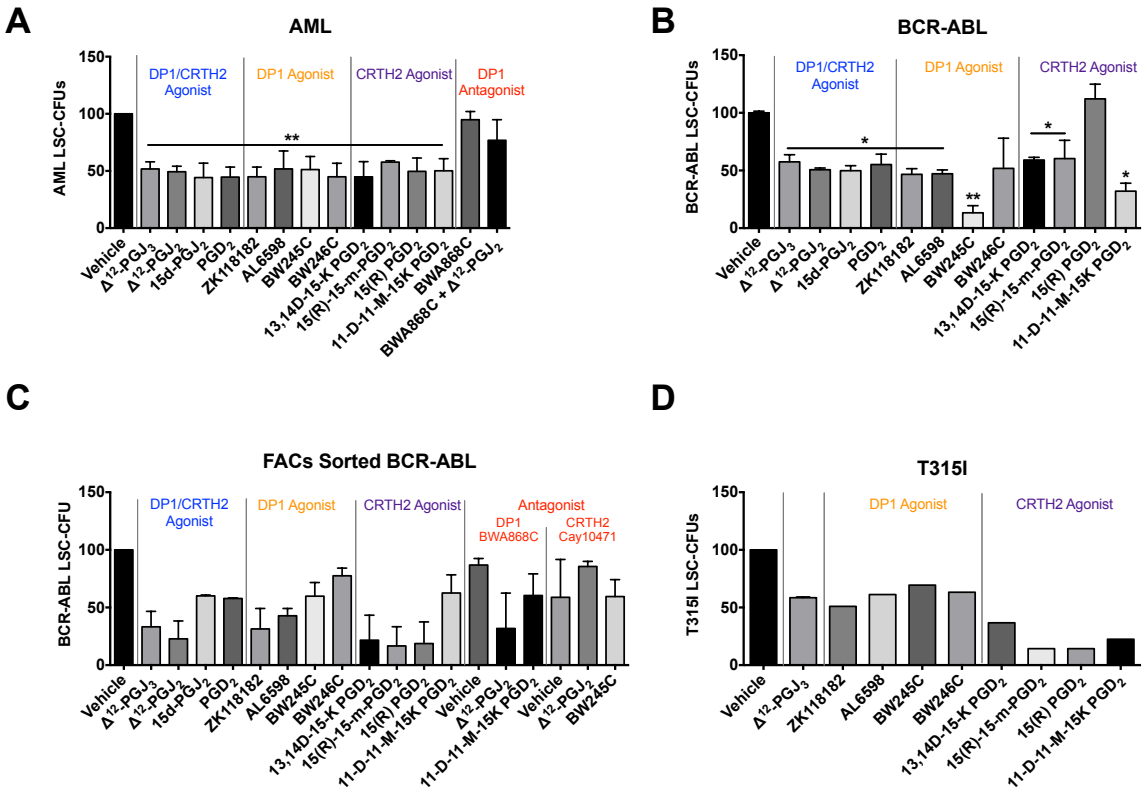

Figure **4-4**: DP receptor agonists decrease LSC-CFUs in three models of experimental leukemia.

Data represent normalized colony counts. In each experiment, DMSO (vehicle: <0.01 % final DMSO) was set as 100%. All other treatments were compared to vehicle control. Treatments were added at time of plating (one dose of treatment) A-C) unsorted splenocytes from leukemic mice were plated. A) MLL-AF9 generated AML. n=3 B) BCR-ABL generated CML. n=3. C) FACS sorted $GFP^{+}c\text{-}kit^{+}sca\text{-}1^{+}$ CML-LSCs. n=2. D) T315I generated CML. n=1-2. Treatments were compared to vehicle control by one-way non-parametric ANOVA. Data represented as experimental mean ± SEM. *p<0.05. **p<0.01.

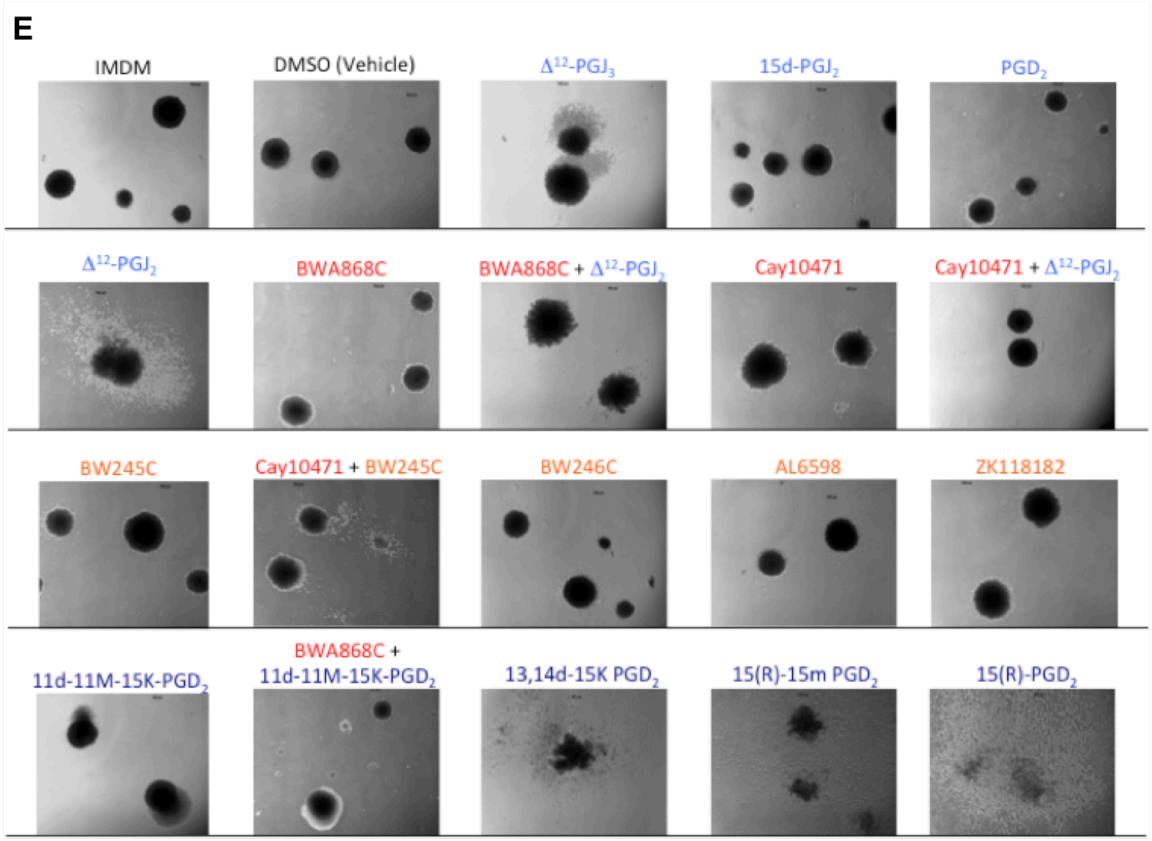

Figure **4-4 (cont)**: DP receptor agonists decrease LSC-CFUs in three models of experimental leukemia.

E) Representative colonies from sorted BCR-ABL LSC colonies. Considerations in counting: size, shape, density, and edge. See IMDM for example).

In vivo activation of the DP receptor

From *in vitro* studies, the most potent DP1 and CRTH2 synthetic agonists were selected to study the *in vivo* effect. In addition to Δ^{12}-PGJ_3, the DP1 agonist BW245C and CRTH2 agonist DK-PGD_2, were utilized in BCR-ABL CML. Beginning on day seven post-transplant, DP receptor agonist treatment (0.05 mg/kg body weight) was administered daily by *i.p.* injection, for seven days. The experimental timeline is shown in Figure 4-5A. After treatment, the blood was analyzed by complete blood count (CBC) for WBC profile. There was a significant reduction in total WBCs in BCR-ABL mice treated with Δ^{12}-PGJ_3, as compared to control (Fig 4-5B). Though both the DP1 agonist (BW245C) and CRTH2 agonist (DK-PGD_2) showed a trend toward decreased WBCs, the difference was not significant. The peripheral blood was also assessed for GFP (which is co-expressed with BCR-ABL). Δ^{12}-PGJ_3 and the CRTH2 agonist (DK-PGD_2) both significantly reduced the circulating BCR-ABL expressing cells (Fig 4-5C). On day 21 post-transplant, mice were euthanized. This day was selected, based on humane endpoints: control and BW245C-treated mice began to show symptoms of CML, with one BW245C mouse succumbing to the disease before it could be euthanized and harvested. The spleen weight, a non-specific indicator of CML burden, was significantly decreased in both Δ^{12}-PGJ_3 and DK-PGD_2 treated mice. As compared to vehicle control, both Δ^{12}-PGJ_3 and DK-PGD_2 treated mice showed a two-fold decrease in spleen weight (Fig 4-5D). LSCs were not different at time of euthanizing mice (data not shown). Treatment period may need to be altered to investigate the LSC-effect. These data suggests CRTH2 activation *in vivo* in BCR-ABL CML mice may reduce total disease burden.

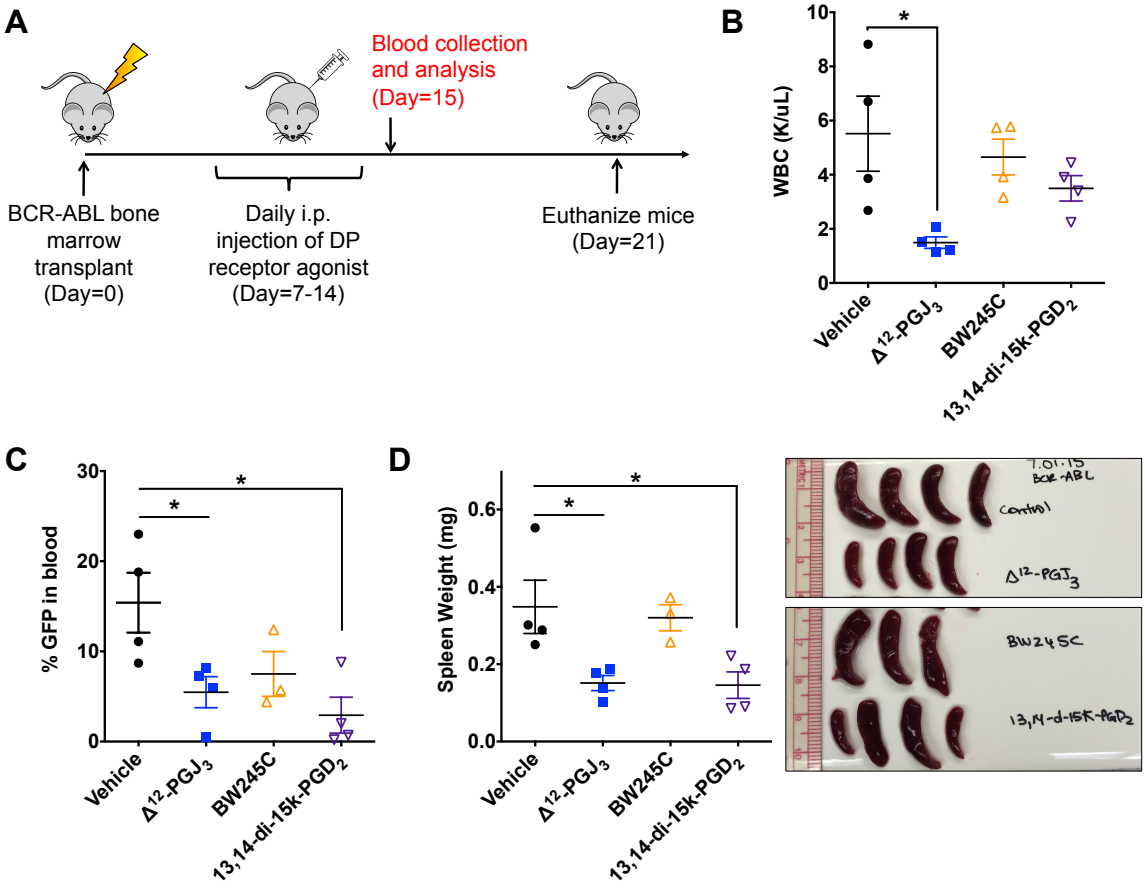

Figure **4-5**: DP receptor agonist treatment reduces BCR-ABL CML burden in mice.

DP receptor agonist treatment (0.05 mg/kg body weight as Δ^{12}-PGJ_3, BW245C, or DK-PGD2) started on day 7 post- BCR-ABL transplant and was administered daily for 7 days. A) Experimental design: D=0 BCR-ABL bone marrow transplant in sub-lethally irradiated mice; days 7-14 post transplant: daily *i.p.* injection of DP receptor agonists; day 15: blood analysis as CBC and flow cytometric GFP; day 21: euthanize based on humane endpoint (DP1 agonist BW245C mouse died and controls were showing severe signs and symptoms). B) Total WBC (K/µl blood) count from CBC analysis of peripheral blood (day 15). n=4 C) Peripheral blood GFP expressed as percentage of gated input (500,000 events collected, gated on FSC). n=4 D) Representative image and spleen weight in BCR-ABL transplanted DP receptor treated mice. n=3-4 D) B-D) all data points shown, with lines and bars representing mean ± SEM. Treatments were compared by ANOVA with multiple comparisons to vehicle. *$p<0.05$.

shRNA knockdown of DP receptor in CML mice

Following the potential preference for Crth2 signaling *in vivo*, shRNA was used to knockdown (KD) the receptor. BCR-ABL LSCs were transduced with Crth2 shRNA. Transduced cells were transplanted and disease was monitored for progression. Interestingly, Crth2 KD BCR-ABL transplantation resulted in lethal disease in ten days. Lin^- splenocytes from Crth2 KD BCR-ABL cells were extracted from mice and treated with Δ^{12}-PGJ_2. Δ^{12}-PGJ_2 treatment in Crth2 KD cells did not decrease the BCR-ABL LSC-CFUs (Fig 4-5A). To confirm the KD of Crth2, protein was harvested from colony assays and whole splenocytes for analysis by Western immunoblotting. Compared to the scramble shRNA control, protein from Crth2 KD colony assay and whole splenocytes showed lower expression of Crth2. There was 24 % reduction in Crth2 in Crth2 KD colony assay cells (Fig 4-5B). This indicated the Crth2 receptor KD prevented the anti-leukemic effect of Δ^{12}-PGJ_2.

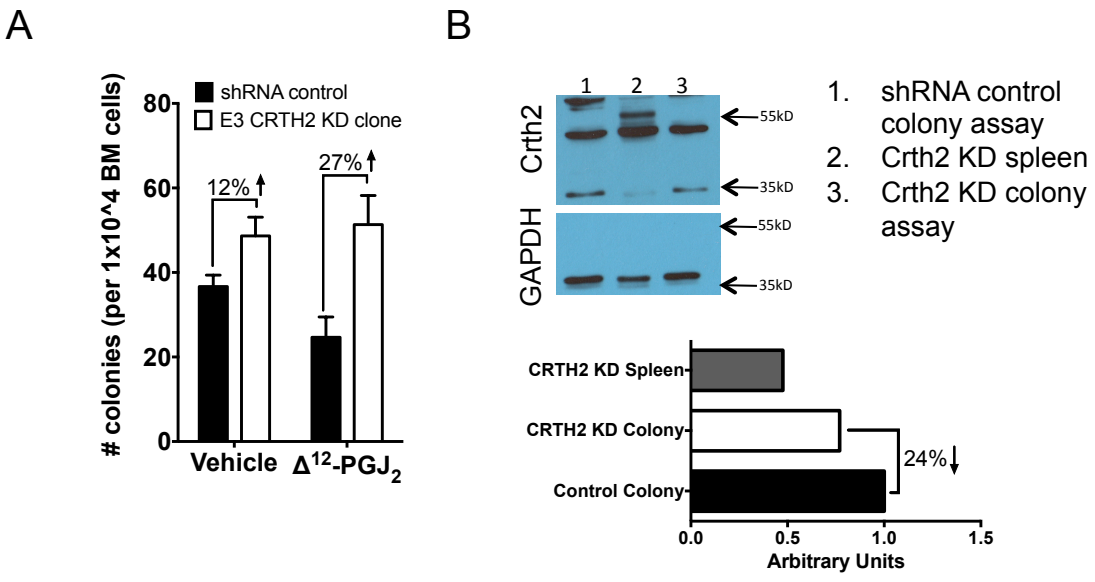

Figure **4-5**: shRNA knockdown of Crth2 in BCR-ABL mice.

BCR-ABL Lin^- cells were transduced with retrovirus containing Crth2 shRNA. Cells were transplanted into mice, and disease progressed to human endpoint. A) Lin^- Crth2 KD (E3 clone) or shRNA control BCR-ABL splenocytes were plated in Methocult with control (<0.01% DMSO) or Δ^{12}-PGJ_2 (100nM). Colonies were counted on day 10. B) Protein expression by western blot analysis of shRNA scramble- BCR-ABL colony assay cells, DP2 (Crth2) KD BCR-ABL colony assay or splenocytes. Western blot analysis with GAPDH loading control; with densitometry shown below. n=2 independent experiments.

Discussion

The DP receptors have a major role in inflammation and allergies. Here, we investigated whether the anti-leukemic effect of CyPGs was mediated through DP receptor binding on LSCs. Though our results suggest the DP receptors may be involved, they do not point to one receptor specifically. In colony assay experiments, with three models of leukemia (MLL-AF9 induced AML; BCR-ABL induced CML; and T315I mutated BCR-ABL), both Dp1 and/or Crth2 activation decreased the formation of LSC-CFUs. There was a pattern of receptor usage that was seen in BCR-ABL CML and T315I mutated BCR-ABL cell cells suggested increased sensitivity to Crth2 signaling. Though the receptors are distinct in terms of their function and ligand-dependent modulation of downstream pathways, it appears in this case, activation of either receptor can impact LSC-CFU formation, though full benefit appears through Crth2 activation.

In a CyPG safety study, we limited our *ex vivo* experiment to basophils. Though expressed on other cell types, both DP1 and CRTH2 are expressed on basophils. Basophil studies did not show activation with agonists known to cause degranulation, specifically PGD_2. This was not a concern. The amount of agonist used in leukemia studies was well below the localized concentrations seen to induce an allergic response. The concentrations were selected based on previous studies showing anti-leukemic functions of CyPGs (1-3, 21). This suggests that *in vivo*,

the proposed CyPG therapy could limit LSCs while not causing excessive inflammation or hypersensitive responses.

Interestingly, there was a difference in the effect of DP receptor activation in BCR-ABL in unsorted splenocytes versus FACS-sorted LSCs, as seen by the ability to form LSC-CFUs. This indicates an important role of the microenvironment in CyPG-mediated elimination of LSCs. One particular DP1 agonist, BW245C, showed significantly reduced LSC-CFUs when unsorted BCR-ABL splenocytes were used (Fig 4-4); while BW245C did not significantly improve disease outcome when used *in vivo* (Fig 4-5). This discrepancy could be due to poor bioavailability or metabolic instability of BW245C *in vivo*, which needs to be further explored. Based on the sorted BCR-ABL-CML LSC data, it appears that CRTH2 activation is more potent than DP1.

In order to conclusively implicate the DP receptors in leukemia, the receptors need to be genetically knocked-out. Along these lines, preliminary data with lentiviral shRNA knockdown of Crth2 in primary BCR-ABL CML cells produced much higher colonies that corroborated well with decreased expression of Crth2. Further studies are currently underway in our laboratory using the CRISPR/Cas9 system, as well as creation of conventional genetic knockout mice, to achieve higher level of knockdown to conclusively prove the role of these receptors. This will be a more definitive way to study the role of these receptors, as synthetic antagonist treatment does not impart a permanent block on signaling, due to competitiveness with endogenous ligands. Based on the experimental data and from mining of the Oncomine database (oncomine.org), it appears that CRTH2, but not DP1, is upregulated in several primary leukemias, including AML and CML. CRTH2 was also upregulated in other cancers, including breast and acute lymphoid leukemia (ALL). Both of these cancers are known to have a distinct chemoresistant cancer stem cell population. Though further mining of this database is required, these data provide some indications towards CRTH2 as a potential target in many cancers (Fig 4-6). Thus, genetic

knockout studies will be prioritized to begin with Crth2 that will be followed-up by knockout of Dp1.

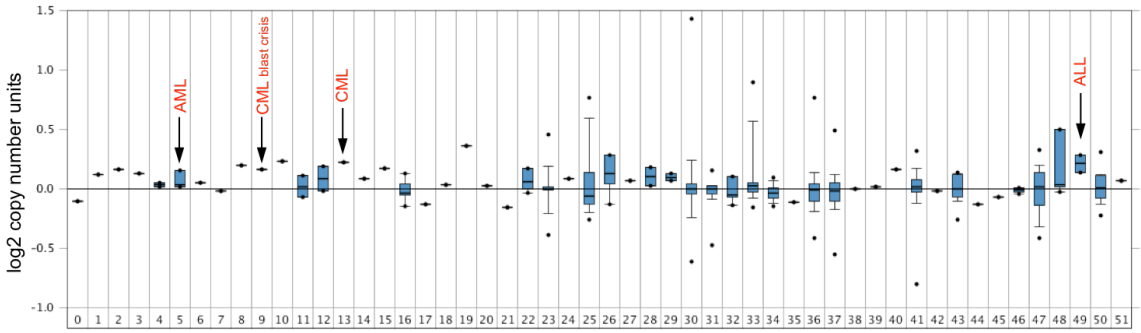

Figure **4-6**: Oncomine analysis of CRTH2 expression in human cancer.

Oncomine data analysis of CRTH2 expression in human cancers. Important samples to note: 0 is no value (healthy control); 1-7 are types of acute leukemias; 9, 13 CML. See Table 4-1 for complete list. Mean above the y=0 axis indicate upregulated CRTH2 expression.

In conclusion, these data suggest CRTH2 may be important in CyPG-mediated LSC signaling. To our knowledge, this is the first time this receptor has been implicated as a potential target to limit cancer. Though more conclusive testing is needed, these findings help to further elicit the mechanism through which CyPGs limit the LSC population. Further studies are warranted to understand how these GCPRs transduce the signal to activate pathways of oxidative stress that ultimately culminate in apoptosis.

References

1. Hegde, S., Kaushal, N., Ravindra, K. C., Chiaro, C., Hafer, K. T., Gandhi, U. H., Thompson, J. T., van den Heuvel, J. P., Kennett, M. J., Hankey, P., Paulson, R. F., and Prabhu, K. S. (2011) Δ12-prostaglandin J3, an omega-3 fatty acid-derived metabolite, selectively ablates leukemia stem cells in mice. *Blood* **118**, 6909-6919
2. Gandhi, U. H., Kaushal, N., Hegde, S., Finch, E. R., Kudva, A. K., Kennett, M. J., Jordan, C. T., Paulson, R. F., and Prabhu, K. S. (2014) Selenium suppresses leukemia through the action of endogenous eicosanoids. *Cancer research* **74**, 3890-3901
3. Finch, E. R., Kudva, A. K., Quickel, M. D., Goodfield, L. L., Kennett, M. J., Whelan, J., Paulson, R. F., and Prabhu, K. S. (2015) Chemopreventive Effects of Dietary Eicosapentaenoic Acid Supplementation in Experimental Myeloid Leukemia. *Cancer prevention research (Philadelphia, Pa.)* **8**, 989-999
4. Hata, A. N., Zent, R., Breyer, M. D., and Breyer, R. M. (2003) Expression and molecular pharmacology of the mouse CRTH2 receptor. *The Journal of pharmacology and experimental therapeutics* **306**, 463-470
5. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M. G. (2000) Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* **403**, 103-108
6. Pérez-Sala, D. (2011) Electrophilic eicosanoids: signaling and targets. *Chemico-biological interactions* **192**, 96-100
7. Ravindra, K. C., Narayan, V., Lushington, G. H., Peterson, B. R., and Prabhu, S. K. (2012) Targeting of Histone Acetyltransferase p300 by Cyclopentenone Prostaglandin Δ 12 -PGJ 2 through Covalent Binding to Cys 1438. *Chemical Research in Toxicology* **25**, 337-347
8. Schuster, V. L. (1998) Molecular mechanisms of prostaglandin transport. *Annual review of physiology* **60**, 221-242
9. Hata, A. N., and Breyer, R. M. (2004) Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacology & therapeutics* **103**, 147-166
10. Boie, Y., Sawyer, N., Slipetz, D. M., Metters, K. M., and Abramovitz, M. (1995) Molecular Cloning and Characterization of the Human Prostanoid DP Receptor. *Journal of Biological Chemistry* **270**, 18910-18916
11. Matsuoka, T., Hirata, M., Tanaka, H., and Takahashi, Y. (2000) Prostaglandin D2 as a mediator of allergic asthma. *Science* **287**, 2013-2017
12. Monneret, G., Gravel, S., Diamond, M., Rokach, J., and Powell, W. S. (2001) Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. *Blood* **98**, 1942-1948
13. Hirata, T., and Narumiya, S. (2012) Prostanoids as regulators of innate and adaptive immunity. *Advances in immunology* **116**, 143-174
14. Nagata, K., Tanaka, K., Ogawa, K., Kemmotsu, K., Imai, T., Yoshie, O., Abe, H., Tada, K., Nakamura, M., Sugamura, K., and Takano, S. (1999) Selective expression of a novel surface molecule by human Th2 cells in vivo. *Journal of immunology (Baltimore, Md. : 1950)* **162**, 1278-1286

15. Hirai, H., Tanaka, K., Yoshie, O., Ogawa, K., Kenmotsu, K., Takamori, Y., Ichimasa, M., Sugamura, K., Nakamura, M., Takano, S., and Nagata, K. (2001) Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *The Journal of experimental medicine* **193**, 255-261
16. Urade, Y., and Hayaishi, O. (1999) Prostaglandin D 2 and sleep regulation. *Biochimica et Biophysica Acta (BBA)* **1436**, 606-615
17. Hayaishi, O., and Urade, Y. (2002) Prostaglandin D2 in sleep-wake regulation: recent progress and perspectives. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* **8**, 12-15
18. Townley, R. G., and Agrawal, S. (2012) CRTH2 antagonists in the treatment of allergic responses involving TH2 cells, basophils, and eosinophils. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **109**, 365-374
19. Kennedy, I., Coleman, R. A., Humphrey, P. P., Levy, G. P., and Lumley, P. (1982) Studies on the characterisation of prostanoid receptors: a proposed classification. *Prostaglandins* **24**, 667-689
20. Krishnamoorthy, S., Recchiuti, A., Chiang, N., Fredman, G., and Serhan, C. N. (2012) Resolvin D1 receptor stereoselectivity and regulation of inflammation and proresolving microRNAs. *The American journal of pathology* **180**, 2018-2027
21. Kudva, A. K., Kaushal, N., Mohinta, S., Kennett, M. J., August, A., Paulson, R. F., and Prabhu, K. S. (2013) Evaluation of the stability, bioavailability, and hypersensitivity of the omega-3 derived anti-leukemic prostaglandin: Δ(12)-prostaglandin J3. *PloS one* **8**(12), e80622
22. Royer, J. F., Schratl, P., Lorenz, S., Kostenis, E., Ulven, T., Schuligoi, R., Peskar, B. A., and Heinemann, A. (2007) A novel antagonist of CRTH2 blocks eosinophil release from bone marrow, chemotaxis and respiratory burst. *Allergy* **62**, 1401-1409
23. Sawyer, N., Cauchon, E., and Chateauneuf, A. (2002) Molecular pharmacology of the human prostaglandin D2 receptor, CRTH2. *British journal of Pharmacology* **137**, 1163-1172
24. Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* **83**, 813-819
25. Wright, H. D., and Metters, K. M. (1998) Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist. *British journal of Pharmacology* **123**, 1317-1324
26. Hawcroft, G., Gardner, S. H., and Hull, M. A. (2004) Expression of prostaglandin D2 receptors DP1 and DP2 by human colorectal cancer cells. *Cancer letters* **210**, 81-84
27. Tippin, B. L., Kwong, A. M., Inadomi, M. J., Lee, O. J., Park, J. M., Materi, A. M., Buslon, V. S., Lin, A. M., Kudo, L. C., Karsten, S. L., French, S. W., Narumiya, S., Urade, Y., Salido, E., and Lin, H. J. (2014) Intestinal tumor suppression in ApcMin/+ mice by prostaglandin D2 receptor PTGDR. *Cancer medicine* **3**, 1041-1051
28. Fukuoka, T., Yashiro, M., Morisaki, T., Kinoshita, H., Hasegawa, T., Kasashima, H., Masuda, G., Sakurai, K., Toyokawa, T., Tanaka, H., Kubo, N., Muguruma, K., Ohira, M., and Hirakawa, K. (2014) The role of type D prostanoid receptors and PPARγ in gastric cancer progression. *Anticancer research* **34**, 2771-2778
29. Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L., and Baltimore, D. (1998) Efficient and

rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* **92**, 3780-3792

30. Pessina, A., Albella, B., Bueren, J., Brantom, P., Casati, S., Gribaldo, L., Croera, C., Gagliardi, G., Foti, P., Parchment, R., Parent-Massin, D., Sibiril, Y., and Van Den Heuvel, R. (2001) Prevalidation of a model for predicting acute neutropenia by colony forming unit granulocyte/macrophage (CFU-GM) assay. *Toxicology in vitro : an international journal published in association with BIBRA* **15**, 729-740
31. Gervais, F. G., Cruz, R. P., Chateauneuf, A., Gale, S., Sawyer, N., Nantel, F., Metters, K. M., and O'Neill, G. P. (2001) Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP. *The Journal of allergy and clinical immunology* **108**, 982-988
32. Ulven, T., and Kostenis, E. (2005) Minor structural modifications convert the dual TP/CRTH2 antagonist ramatroban into a highly selective and potent CRTH2 antagonist. *Journal of medicinal chemistry* **48**, 897-900

Table **4-1**: Oncomine Legend

Legend	
0. No value (1)	26. Invasive Ductal Breast Carcinoma (4)
1. Acute Biphenotypic Leukemia (1)	27. Leiomyosarcoma (1)
2. Acute Lymphoblastic Leukemia (1)	28. Liposarcoma (2)
3. Acute Monoblastic Leukemia (1)	29. Lung Adenocarcinoma (2)
4. Acute Monocytic Leukemia (2)	30. Medulloblastoma (125)
5. Acute Myeloid Leukemia (6)	31. Melanoma (33)
6. Acute Myelomonocytic Leukemia (1)	32. Meningioma (6)
7. Acute Promyelocytic Leukemia (1)	33. Myelodysplastic Syndrome (19)
8. Biphasic Synovial Sarcoma (1)	34. Neuroglial Tumor, NOS (28)
9. Blast Phase Chronic Myelogenous Leukemia (1)	35. Non-Hodgkin's Lymphoma (1)
10. Brain Glioblastoma (1)	36. Non-Small Cell Lung Carcinoma (196)
11. Breast Adenocarcinoma (2)	37. Ovarian Carcinoma (96)
12. Breast Carcinoma (2)	38. Pleomorphic Hepatocellular Carcinoma (1)
13. Chronic Myelogenous Leukemia (1)	39. Pleomorphic Liposarcoma (1)
14. Colon Adenocarcinoma (1)	40. Prostate Adenocarcinoma (1)
15. Colon Carcinoma (1)	41. Prostate Carcinoma (36)
16. Colorectal Carcinoma (8)	42. Prostate Small Cell Carcinoma (1)
17. Dedifferentiated Liposarcoma (1)	43. Renal Carcinoma (14)
18. Desmoplastic Medulloblastoma (1)	44. Renal Cell Carcinoma (1)
19. Ductal Breast Carcinoma (1)	45. Sarcoma (1)
20. Endometrial Carcinoma (1)	46. Schwannoma (5)
21. Erythroleukemia (1)	47. Small Cell Lung Carcinoma (18)
22. Esophageal Squamous Cell Carcinoma (4)	48. Synovial Sarcoma (4)
23. Gastrointestinal Stromal Tumor (22)	49. T-Cell Acute Lymphoblastic Leukemia (2)
24. Glioblastoma (1)	50. Thyroid Gland Carcinoma (19)
25. Hepatocellular Carcinoma (14)	51. Uveal Melanoma (1)

Chapter 5

Conclusions and Future Directions

Conclusions

Three questions were addressed in this dissertation to examine the central hypothesis that ***endogenous CyPG production can be specifically enhanced through dietary manipulation and that the endogenous CyPGs produced are able to limit the LSC population, resulting in a lesser disease burden***.

1. Does supplementation with pharmacological doses of EPA increase endogenous Δ^{12}-PGJ_3 to levels to eliminate LSCs in experimental models of myeloid leukemias?
2. Is PPARγ activation required for the anti-leukemic effect of selenium supplementation, which is mediated through endogenous CyPGs?
3. Do CyPGs eliminate LSCs through binding to DP receptors?

Based on the experimental data collected in response to the above-mentioned questions, the major conclusions from each data chapter are summarized below:

Chapter 2. Does supplementation with pharmacological doses of EPA increase endogenous Δ^{12}-PGJ_3 to levels able to eliminate LSCs in models of myeloid leukemias?

In the first data chapter, pharmacological levels of EPA (eicosapentaenoic acid) were fed to mice as part of the diet. The supplementation was energetically equivalent to the FDA approved drug Lovaza®, which is clinically used to treat hyperlipidemia. In mice, this equated to 1.8 % of total energy: a portion of the lipid content (as corn oil) was replaced with either EPA or oleic acid (OA), as control. The phospholipid membrane in RBCs, spleen, and liver were enriched with omega-3, as shown by omega-3 index, following eight weeks on EPA-supplemented diet. Our data suggest omega-3 supplementation affects the composition of each tissue in a unique

manner. The overall content of omega-3 is important, but the fold increase of the omega-3 index with EPA-supplementation was of great interest. However, the trend of an increase is just as important, if not more, than the absolute content in a particular tissue.

Though tissue distribution of omega-3 was not a central focus of our studies, the content of omega-3 in each tissue follows basic understanding of lipid metabolism. The liver is known to be central in lipid metabolism and dietary lipid accumulation is expected. It follows that the highest overall highest omega-3 index was in the liver of EPA-supplemented mice: EPA-supplemented liver showing an omega-3 index of 20 % versus 8 % omega-3 seen in OA-supplemented liver, a 2.5-fold increase. The spleen is a secondary lymphatic organ and can be thought of as a filter for circulating cells. In the spleen EPA-supplementation lead to an omega-3 index of 11 %, where OA-supplementation showed 3 %, a 3.5-fold increase. Though the actual content was the lowest, the omega-3 index was most affected in RBCs: EPA-supplementation increased the omega-3 index over five-fold: from 1 % in OA-supplemented mice to 5.5 % in EPA-supplemented mice.

The increased omega-3 index observed with EPA-supplementation was associated with increased production Δ^{12}-PGJ_3. Healthy EPA-supplemented mice, when treated with LPS, produced more endogenous Δ^{12}-PGJ_3, quantified from serum lipids 12 hours post-LPS treatment (1 mg/kg body weight). Increased Δ^{12}-PGJ_3 production with EPA-supplementation was sufficient to limit leukemia burden. Specifically, the leukemia stem cell (LSC) population was significantly decreased in EPA-supplemented mice, in three models of myeloid leukemia. The anti-leukemic effect was attributed to EPA-derived Δ^{12}-PGJ_3, a cyclopentenone prostaglandin (CyPG). Inhibition of cyclooxygenase (COX) *in vivo* revered the EPA-mediated protective effect. This suggests a prostanoid derived from EPA as being the essential agent. However, as COX leads to a broad class of compounds, a specific hematopoietic prostaglandin D-synthase (H-PGDS), HQL-79, was also administered to EPA-supplemented BCR-ABL mice. Again, the protective effect of

EPA was reversed. As omega-3 supplementation produces unique anti-inflammatory prostaglandins in general, these data suggest high-dose EPA as a beneficial supplement in leukemia patients. ***EPA-supplementation leads to sufficient production of Δ^{12}-PGJ$_3$; enough endogenous Δ^{12}-PGJ$_3$ is produced to limit the LSC population, in two models of CML and an AML model.***

Chapter 3. Is PPARy activation required for the anti-leukemic effect of selenium supplementation, which is mediated though endogenous CyPGs?

Selenium supplementation shifts the prostaglandin cascade from pro-inflammatory PGs towards more anti-inflammatory CyPGs via the differential expression of several downstream PG synthases, including the prostaglandin D synthase (PGDS; hematopoietic-type). These CyPGs, namely Δ^{12}-PGJ$_2$ and 15d-PGJ$_2$, are known agonists for PPARγ. Recently, PPARγ activation with synthetic agonist, pioglitazone, decreased the LSC population (1). Here, supplementation of CML mice with supraphysiological, but non-toxic doses, of selenium (at 0.4 ppm) limited the disease with low traces of LSCs. However, use of GW9662, a PPARγ antagonist, in selenium supplemented CML mice blocked the anti-leukemic effect of selenium. Not only did GW9662 inhibit PPARγ, but also increase several regulators of "stem-like" properties, including STAT5a, HIF2α, and CITED2. An unexpected result that needs further study is that 15-PGDH was upregulated with GW9662 treatment. This was accompanied by increased concentration of 13,14-dihydro-15-keto-PGJ$_2$, a prostaglandin-derivative never before detected *in vivo*. Of note, CML was not completely inhibited with selenium supplementation alone and pioglitazone treatment was also not completely protective. This emphasizes that a dual therapy, with one targeting the bulk cells (TKIs) and the other targeting the LSCs (selenium and/or pioglitazone) is necessary to

completely eradicate the disease. However our data suggest that ***selenium supplementation in experimental CML leads to production of endogenous CyPGs, namely Δ^{12}-PGJ$_2$ and 15d-PGJ$_2$, which activate PPARγ, thus limiting the LSC population.***

Chapter 4. Do CyPGs eliminate LSCs through binding to DP receptors?

The LSC-progenitor population was decreased both *in vitro* (decreased LSC-colony forming units) and *in vivo,* when DP receptors were activated with CyPGs and other synthetic receptor agonists. The CRTH2 receptor was specifically indicated, as both antagonist treatment and shRNA knockdown limited the anti-leukemic effect. Most studies have been conducted in BCR-ABL mice, which is the reason for more extensive investigation of the DP receptor expression in these mice. It appears that enrichment of LSCs also enriches for the DP receptor, especially Crth2. ***Though more studies are needed to fully understand the mechanism, DP receptor binding of the CyPGs, Δ^{12}-PGJ$_2$ and Δ^{12}-PGJ$_3$, limits the LSC population, in models of myeloid leukemias.***

Overall, this dissertation improved our understanding of anti-leukemic CyPGs. Dietary supplementation of EPA and/or selenium changed the production of endogenous CyPGs where more anti-inflammatory and anti-leukemic CyPGs, including Δ^{12}-PGJ$_2$ and Δ^{12}-PGJ$_3$, were produced. It appears that both LSCs and nearby cells, including macrophages, can generate these CyPGs. A full understanding of how these compounds enter LSCs still needs further research. More studies are needed to understand why the size of LSC colonies was affected by various treatments. This can be done with gene expression analysis for progenitor markers, which will allow for more definitive classification of the cells. Additionally what levels of endogenous CyPGs that are produced in the LSCs, as compared to neighboring cells, is not clear. As both

inhibition of the surface GPCRs and of PPARγ limited the anti-leukemic effects, it appears that CyPGs act through a dual-mechanism in LSCs. The normally quiescent LSC phenotype becomes more metabolically activated with CyPGs. This results in LSC- differentiation and/or induction of apoptosis. Further studies are still needed to fully understand this mechanism, and to translate these findings to a clinical setting.

Limitations of Research

Though every effort was made in the design and execution of experiments, there are limitations that should be addressed. Below are a few of these limitations, which should be considered when making a statement about what the presented data could mean for translation to a clinical setting.

1. Experimental leukemia model:

   Three types of myeloid leukemia were studied: BCR-ABL, T315I, and MLL-AF9. Each transplant had the potential for being different, which could explain the variation seen in some experiments. Though cells were counted and equally distributed into syringes, retro-orbital injection leaves room for human error. The biological sample size was also limited in some cases, due to the cost of the diet. This made it especially important that all retro-orbital transplants were done perfectly. Additionally, as the dietary effect was of greatest interest, the fact that mice were group housed together and that feed was given ad libitum for the cage of mice, there was no way to ensure that all mice were eating enough of the feed. If the dietary lipids and/or selenium were not sufficient in a particular mouse, this would also influence the results.

2. Omega-3 index:

The increased omega-3 index in EPA-supplemented mice is central in the ability of mice to produce Δ^{12}-PGJ_3. However, all omega-3 index studies were done in healthy mice. The omega-3 index was not measured following irradiation or in mice transplanted with leukemic bone marrow.

3. Tyrosine kinase inhibitors:

   Tyrosine kinase inhibitor (TKI) therapy was mentioned as being the standard of care for CML. We also made the statement that TKI therapy in conjunction with the discussed dietary manipulation could potentially eradicate leukemia, as the TKI would target the bulk cells and the endogenous CyPGs would target LSCs. However, we did not actually carry out any of these studies. Previous work from our laboratory allowed for this bold statement, but further research is needed in order to confirm this hypothesis. As an example of how these experiments could be conducted: in EPA- or OA-supplemented mice, TKI therapy would be administered one-week following bone-marrow transplant with BCR-ABL. The TKI treatment would be given for seven days. Blood analysis by CBC before, during, and after TKI treatment would be done to monitor the TKI-effect. It is expected that EPA-supplemented mice would be cured of CML following this short TKI treatment, as the EPA-derived CyPGs would eliminate LSCs and the TKI would eliminate bulk tumor cells. OA-supplemented mice would have decreased WBCs and disease during TKI treatment, but this protective effect would be lost when TKI treatment was removed.

Future Directions

The questions asked herein were designed based on the results of previous studies from our laboratory that selenium impacts endogenous production of anti-leukemic CyPGs.

Experiments were designed for the potential for translation to humans. However, as primarily mouse models of leukemia were used, these findings must be tested in a clinical setting in order to fully understand the impact. Additionally, the mechanism of anti-leukemic CyPGs needs to be further studied, to determine whether this therapy has the potential to eliminate other stem cell populations in other cancers or if this restricted to only myeloid leukemias.

Translational future directions

1. SNPs in prostanoid enzymes and relationship with leukemias

Some reports show an association between COX overexpression and cancer development (2, 3). However, in our studies, high COX-2 activity is essential for the endogenous production of anti-leukemic CyPGs, particularly with EPA as the substrate. GWAS studies to determine if there is a relationship between leukemias and SNPs in prostanoid machinery are important to translate these findings to humans. SNPs in COX-2 have been related to increased risk of other cancers, including breast cancer (4), colorectal cancer (5), and liver cancer (6). However, only one study of 266 cases of childhood leukemias as acute lymphoblastic leukemia (ALL), showed a SNP in COX-2 was slightly associated with childhood ALL (p=0.06) (7). To look more specifically at the pathway described in this dissertation, the expression of H-PGDS and levels of Δ^{12}-PGJ_2 should also be studied in clinical samples. Interestingly, intestinal tumors in APC(Min/+) mice were suppressed by over 80 % when H-PGDS was expressed (8). SNPs in DP receptors, PPARγ, and NF-κB should also be studied, as this entire pathway is important in the anti-leukemic properties of the CyPGs discussed.

2. Diet and leukemia

In 1997, the World Cancer Research Fund and the American Institute for Cancer Research estimated that 30-40 % of all cancers could be prevented my modifying lifestyle factors like diet and exercise (9, 10). Dietary patterns have been studied through prospective studies in the context of leukemia risk, but with little consistency in results between studies. Some studies show an inverse risk of adult leukemia with vegetable consumption (11). Though it could be a stretch, this could indicate increased selenium-enriched vegetables could result in reduced leukemia risk, as selenium intake is largely based on the soil concentration. A case-control leukemia study showed that though cases and controls ate the same amount of fish, total fat intake was lower in controls. This indicates the omega-6:omega-3 ratio, or increased proportion of total energy from fish, as being important in leukemia prevention (12). The European Prospective Investigation into Cancer and Nutrition (EPIC) trial, a prospective cohort study, showed no link between diet and leukemia (10).

From our studies, it appears that supplemental levels of EPA and/or selenium can prevent leukemias through elimination of LSCs. However, these studies were done in highly controlled mouse models. In order to translate our results, intervention studies are needed. In the case of omega-3 supplementation, there is already an FDA approved drug, Lovaza®. In a pilot study, Lovaza® lead to detectable levels of Δ^{12}-PGJ_3 in human serum. This indicates the potential translatability of our studies. Lovaza®, in conjunction with TKI therapy, should be given to newly diagnosed CML patients. As EPA-derived PGs are beneficial in settings other than leukemia, including inflammation and cardiovascular health, high-dose omega-3 intervention may have a greater overall impact compared with selenium supplementation. As excessive selenium can be harmful, and has been linked with obesity, and diabetes, the dietary intervention would be more complex. Additionally, some individuals require more selenium than others. Understanding what supplemental intervention would be best for each individual would require genetic screening of SNPs in genes discussed above. The complexity of translating basic science

to clinical interventions has been a limitation in the success of many chemoprevention research studies (13).

3. Allergies and leukemia

Our studies implicated the DP receptors are important in the anti-leukemic effect of CyPGs. As the DP receptors are usually studied in the context of allergies, it would be interesting to see if there is a correlation between the use of anti-allergics and incidences of leukemia. Asparaginase-treatment for ALL has been associated with development of allergies. Interestingly, GPCRs were implicated as possibly contributing to asparaginase-hypersensitivity, though the specific receptors were not described (14).

4. Insulin resistance and leukemia

Insulin resistance is associated with both type-2 diabetes and cancer. PPARγ activation, with pioglitazone, is a therapy for type-2 diabetics and experimentally reduces CML LSCs (1). In a recent paper, mathematical modeling of the insulin-signaling network (ISN) investigated how ISN proteins regulate cell cycle progression and cell death (15). There could be a connection with our data. How do CyPGs fit into this pathway? Are individuals with limited ability to make CyPGs at higher risk for type-2 diabetes?

NSAID treatment, specifically, aspirin, has been implicated as preventative in insulin resistance (16): it is hypothesized that insulin resistance arises from low-grade systemic inflammation, from activation of NF-κB. However, from our data, not all prostaglandins are created equal in terms of their inflammatory capacity. Additionally, NF-κB inhibition is a central mechanism of CyPG-dependent reduction of inflammation. Research on EPA-derived CyPGs (and/or selenium shunting) in the context of insulin resistance should be studied. It is possible that

limiting ARA-derived PGs by increasing EPA in the membrane could limit low-grade inflammation associated with both insulin resistance and cancer development.

Laboratory future directions

1. What is the function of 15-PGDH (15-hydroxy-PG dehydrogenase) upregulation, and the resulting prostaglandins, when PPARγ is inhibited *in vivo*?

In Chapter 3, there was an increase in levels of 13,14-dihydro-15-keto-PGJ_2 in Se-S BCR-ABL mice treated with the PPARγ inhibitor, GW9662. We assumed this was a compensatory mechanism through which natural PPARγ ligands were broken-down. However, this was the first time 13,14-dihydro-15-keto-PGJ_2 production has been shown *in vivo*. The biological activity of this compound is unknown, as is the D series derivative (13,14-dihydro-15-keto-PGD_2). However, in Chapter 4, 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2) was able to reduce LSC-CFUs *in vitro*, and limit CML burden *in vivo*. This indicates that this compound, which has previously all but been discounted as having biological activity, may have anti-leukemic properties. Is this through binding to PPARγ or DP receptor activation? Or does DK-PGD_2 have an unknown mechanism of action? The same may also be true for 13,14-dihydro-15-keto-PGJ_2.

Additionally, up-regulation of 15-PGDH, which oxidizes many prostaglandins, with GW9662 is an interesting finding that needs to be further explored. From our lab, 15-PGDH has been shown to be upregulated with selenium supplementation in dextran sodium sulfate-colitis. In this model, upregulation was protective, as 15-PGDH-dependent oxidation of PGE_2 helps to alleviate inflammation (17). This emphasizes tissue-specific function of prostaglandins and enzymes associated with eicosanoid metabolism.

Of note, the J-family PGs were only recently accepted as having biological activity *in vivo*, with some scientists still believing they are simply a non-functional breakdown product. This highlights the unknown territory of dehydrated PGs in biological response. The limitation of studying these products is their short half-life, their binding to proteins, such as serum albumin *in vivo*, and the seemingly endless possibility of derivatives. Collaboration between basic chemists and biologists is necessary to propel this field forward.

2. NOD/SCID mice for xenograft of clinical samples

Patient-derived TKI resistant CML LSCs should be transplanted into NOD/SCID mice on purified EPA and/or selenium diet. Additionally, TKI + diet therapy should be studied *in vivo*/ *in vitro* to see if all CML cells can be eliminated by combination therapy. Additionally, p53 activation has been implicated in the CyPG-mediated effect. As p53 is mutated in many cancers, including some cases of AML, this mutation should be further studied in patient-derived leukemia samples. Use of clinical samples (with known p53 mutations) should help to understand if the mechanism of CyPG-induced LSC-elimination. It is still unclear whether the elimination of LSCs is through p53-mediated apoptosis alone, or if the network of signaling is more complex, as it appears that several regulatory pathways are involved.

3. DP receptor in LSCs

Though our data support DP receptor binding on LSCs, this pathway must be further clarified. Ongoing research using CRISPER/Cas9 knockout and generation of conventional genetic knockout mice will allow for understanding of the essentiality of this receptor family. It is also possible that CyPGs act through several mechanisms in the context of leukemia, including

PPARγ-NF-κB balance. It will be interesting to see if there is a compensatory mechanism in place when DP receptor signaling in eliminated as a possibility.

We have demonstrated deregulation of metabolic signaling and increased ROS as mediated through PPARγ activation (inhibition of STAT5a, HIFα, and CITED2), inhibition of NF-κB, and activation of p53, however, how does the DP receptor fit into this proposed cascade?

In immune cells the DP1 receptor is associated with a Gαs subunit, which signals and increases intracellular cAMP and phosphokinase A (PKA). A recent study looked at the role of Gαs in stem cell fate decisions, in context of PKA signaling. When the Gαs subunit was deleted, the stem cell compartment was significantly expanded and resulted in carcinogenesis (in this case, basal-cell carcinogenesis). This study indicates that Gαs-PKA signaling is important in the proliferation of stem cells (18). Another study showed that the upregulation of ROS/HIF is essential for upregulation of PKA during hypoxia (19). As quiescent LSCs have highly regulated ROS, this finding could mean there is a link between HIF and PKA, linking activation of DP1 to influencing HIFs, which are central in the maintenance of the LSC population.

CRTH2 is associated with a Gαi subunit, leading to increased Ca^{2+}, and activation of phospholipase D (PLD). In an *in vitro* study, down-regulation of PLD, induced senescence in the colon cancer cell line HCT-116. Interestingly, in p53-mutated cells, this did not occur (20). Though this is a somewhat convoluted connection, it emphasizes the complexity of cellular signaling, and could highlight a connection between CRTH2 binding and p53 activation in leukemic cells.

References

1. Prost, S., Relouzat, F., Spentchian, M., Ouzegdouh, Y., Saliba, J., Massonnet, G., Beressi, J.-P. P., Verhoeyen, E., Raggueneau, V., Maneglier, B., Castaigne, S., Chomienne, C., Chrétien, S., Rousselot, P., and Leboulch, P. (2015) Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature* **525**, 380-383
2. Gupta, A., Huet, Y. M., and Dey, S. K. (1989) Evidence for prostaglandins and leukotrienes as mediators of phase I of estrogen action in implantation in the mouse. *Endocrinology* **124**, 546-548
3. Lu, Q.-Y. Y., Jin, Y., Mao, J. T., Zhang, Z.-F. F., Heber, D., Dubinett, S. M., and Rao, J. (2012) Green tea inhibits cycolooxygenase-2 in non-small cell lung cancer cells through the induction of Annexin-1. *Biochemical and biophysical research communications* **427**, 725-730
4. Dai, Z.-J. J., Shao, Y.-P. P., Ma, X.-B. B., Xu, D., Tang, W., Kang, H.-F. F., Lin, S., Wang, M., Ren, H.-T. T., and Wang, X.-J. J. (2014) Association of the three common SNPs of cyclooxygenase-2 gene (rs20417, rs689466, and rs5275) with the susceptibility of breast cancer: an updated meta-analysis involving 34,590 subjects. *Disease markers* **2014**, 484729
5. Li, H., Guo, Q., Zhou, B., and He, S. (2015) Cyclooxygenase-2 gene polymorphisms and the risk of colorectal cancer: A population-based study. *Oncology letters* **10**, 1863-1869
6. Chen, Z., Zhu, J., Huang, C., Lian, F., Wu, G., and Zhao, Y. (2015) The association between three cyclooxygenase-2 polymorphisms and hepatocellular carcinoma risk: a meta-analysis. *PloS one* **10**(3) e0118251
7. Wang, C.-H. H., Wu, K.-H. H., Yang, Y.-L. L., Peng, C.-T. T., Wang, R.-F. F., Tsai, C.-W. W., Tsai, R.-Y. Y., Lin, D.-T. T., Tsai, F.-J. J., and Bau, D.-T. T. (2010) Association study of cyclooxygenase 2 single nucleotide polymorphisms and childhood acute lymphoblastic leukemia in Taiwan. *Anticancer research* **30**, 3649-3653
8. Park, J., Kanaoka, Y., Eguchi, N., Aritake, K., Grujic, S., Materi, A. M., Buslon, V. S., Tippin, B. L., Kwong, A. M., and Salido, E. (2007) Hematopoietic prostaglandin D synthase suppresses intestinal adenomas in ApcMin/+ mice. *Cancer research* **67**, 881-889
9. Glade, M. J. (1999) Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997. *Nutrition (Burbank, Los Angeles County, Calif.)* **15**, 523-526
10. Saberi Hosnijeh, F., Peeters, P., Romieu, I., Kelly, R., Riboli, E., Olsen, A., Tjønneland, A., Fagherazzi, G., Clavel-Chapelon, F., Dossus, L., Nieters, A., Teucher, B., Trichopoulou, A., Naska, A., Valanou, E., Mattiello, A., Sieri, S., Parr, C. L., Engeset, D., Skeie, G., Dorronsoro, M., Barricarte, A., Sánchez, M.-J. J., Ericson, U., Sonestedt, E., Bueno-de-Mesquita, H. B., Ros, M. M., Travis, R. C., Key, T. J., Vineis, P., and Vermeulen, R. (2014) Dietary intakes and risk of lymphoid and myeloid leukemia in the European Prospective Investigation into Cancer and Nutrition (EPIC). *Nutrition and cancer* **66**, 14-28
11. Ross, J. A., Kasum, C. M., Davies, S. M., Jacobs, D. R., Folsom, A. R., and Potter, J. D. (2002) Diet and risk of leukemia in the Iowa Women's Health Study. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **11**, 777-781

12. Fritschi, L., Ambrosini, G. L., Kliewer, E. V., Johnson, K. C., and Group, C. (2004) Dietary fish intake and risk of leukaemia, multiple myeloma, and non-Hodgkin lymphoma. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **13**, 532-537
13. Potter, J. D. (2014) The failure of cancer chemoprevention. *Carcinogenesis* **35**, 974-982
14. Chen, S. H. H., Pei, D., Yang, W., Cheng, C., Jeha, S., Cox, N. J., Evans, W. E., Pui, C. H. H., and Relling, M. V. (2010) Genetic variations in GRIA1 on chromosome 5q33 related to asparaginase hypersensitivity. *Clinical pharmacology and therapeutics* **88**, 191-196
15. Bertuzzi, A., Conte, F., Mingrone, G., Papa, F., Salinari, S., and Sinisgalli, C. (2016) Insulin Signaling in Insulin Resistance States and Cancer: A Modeling Analysis. *PloS one* **11**(5), e0154415
16. Amiri, L., John, A., Shafarin, J., Adeghate, E., Jayaprakash, P., Yasin, J., Howarth, F. C., and Raza, H. (2015) Enhanced Glucose Tolerance and Pancreatic Beta Cell Function by Low Dose Aspirin in Hyperglycemic Insulin-Resistant Type 2 Diabetic Goto-Kakizaki (GK) Rats. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **36**, 1939-1950
17. Kaushal, N., Kudva, A. K., Patterson, A. D., Chiaro, C., Kennett, M. J., Desai, D., Amin, S., Carlson, B. A., Cantorna, M. T., and Prabhu, K. S. (2014) Crucial role of macrophage selenoproteins in experimental colitis. *Journal of immunology (Baltimore, Md. : 1950)* **193**, 3683-3692
18. Iglesias-Bartolome, R., Torres, D., Marone, R., Feng, X., Martin, D., Simaan, M., Chen, M., Weinstein, L. S., Taylor, S. S., Molinolo, A. A., and Gutkind, J. S. (2015) Inactivation of a Gα(s)-PKA tumour suppressor pathway in skin stem cells initiates basal-cell carcinogenesis. *Nature cell biology* **17**, 793-803
19. Shaikh, D., Zhou, Q., Chen, T., Ibe, J. C., Raj, J. U., and Zhou, G. (2012) cAMP-dependent protein kinase is essential for hypoxia-mediated epithelial-mesenchymal transition, migration, and invasion in lung cancer cells. *Cellular signalling* **24**, 2396-2406
20. Lee, Y.-H. H., and Bae, Y.-S. S. (2014) Phospholipase D2 downregulation induces cellular senescence through a reactive oxygen species-p53-p21Cip1/WAF1 pathway. *FEBS letters* **588**, 3251-3258

VITA
Emily Rebecca Finch

Education:

2011-present	The Pennsylvania State University Ph.D. in Immunology and Infectious Diseases: GPA 3.99/4.00 Advised by Dr. K. Sandeep Prabhu: GPA: 3.99
2013-2014	Penn State Hershey College of Medicine Graduate Certificate in Translational Sciences: GPA 4.00/4.00
2006-2011	The Pennsylvania State University Bachelors of Science in Nutrition, Basic Science Option: GPA 3.62/4.00 Bachelors of Science in Science, Life Science Option: GPA 3.62/4.00

Publications:

- **E. R. Finch**, A. K. Kudva, M. D. Quickel, L. L. Goodfield, M. J. Kennett, J. R. Whelan, R. F. Paulson, K. S. Prabhu. Chemopreventive effects of dietary eicosapentaenoic acid supplementation in experimental myeloid leukemia. *Cancer Prev Res* 2015, 8; 989-999**.** *Selected for highlight in Nutrition Frontiers, spring 2016.*
- U. H. Gandhi, N. Kaushal, S. Hegde, **E. R. Finch*,** A. K. Kudva, M. Kennett, C. Jordan, R. F. Paulson, and K. S. Prabhu. Selenium suppresses leukemia through the action of endogenous eicosanoids. *Cancer Res.* July 15, 2014, 74; 3890. *2nd author.
- D. B. Tukaram*, **E. R. Finch*,** C. Liao*, A. E. Shay*, K. S. Prabhu (2016). The role of selenoproteins in the resolution of inflammation, Chap 42. Selenium: its molecular biology and role in human health. Editors: Hatfield D, Gladyshev VN, Scheiwzer U, Carlson BA. Springer, NY. Submitted. **Authors contributed equally.*

Professional Presentations:

- The role of endogenous lipid mediators in models of experimental leukemia. 16th International Winter Eicosanoid Conference. March 13-16, 2016, Baltimore, Maryland. **Invited Presentation.*
- The role of eicosapentaenoic acid in models of experimental leukemia. Presented at the 14th International Conference on Bioactive Lipids in Cancer, Inflammation, and Related Diseases. July 12-15, 2015, Budapest, Hungary. **Outstanding Young Scientist Nominee.*
- The role of dietary omega-3 fatty acid in experimental leukemia. Presented at the 2014 American Institute for Cancer Research Annual Research Conference, October 29-31, 2014, Washington DC. **AICR Scholarship Winner.*
- Effect of a diet-derived eicosapentaenoic acid metabolite in a rodent model of CML. Presented at the 16th Annual John Goldman Conference on Chronic Myeloid Leukemia: Biology and Therapy. September 4-7, 2014, Philadelphia, Pennsylvania,
- Effect of an eicosapentaenoic acid derivative in a rodent model of leukemia. Presented at the 13th International Conference on Bioactive Lipids in Cancer, Inflammation, and Related Diseases. November 3-6, 2013, San Juan, Puerto Rico.
- The effect of high dietary eicosapentaenoic acid supplementation in leukemic mice. Presented at Experimental Biology: American Society of Nutrition Meeting. April 18-24, 2013, Boston, Massachusetts.

Honors and Awards:

- Santosh Nigam Memorial Outstanding Young Scientist Award Nominee, 2015
- J. Lloyd and Dorothy Foehr Huck Endowment Travel Stipend, 2015
- American Institute for Cancer Research (AICR) scholarship to attend the 2014 AICR Annual Research Conference, 2014
- Clinical and Translational Science Institute TL1 Scholar, 2013-2014
- Sahakian Family Endowment Travel Award, 2013
- College of Agricultural Sciences Competitive Grant, The Pennsylvania State University, 2013
- Graduate Enrichment Fund, Huck Institutes of the Life Sciences, The Pennsylvania State University, 2013