DISSECTING RECEPTORS AND SIGNALING PATHWAYS DRIVING ALL-*TRANS* RETINOIC ACID-INDUCED DIFFERENTIATION OF THE ACUTE MYELOID LEUKEMIA CELL LINE, HL-60: IN PURSUIT OF ACTIONABLE TARGETS FOR EXPANSION OF DIFFERENTIATION THERAPY

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Robert James MacDonald

May 2018

DISSECTING RECEPTORS AND SIGNALING PATHWAYS DRIVING ALL-*TRANS* RETINOIC ACID-INDUCED DIFFERENTIATION OF THE ACUTE MYELOID LEUKEMIA CELL LINE, HL-60: IN PURSUIT OF ACTIONABLE TARGETS FOR EXPANSION OF DIFFERENTIATION THERAPY

Robert James, MacDonald, Ph. D.

Cornell University 2018

Differentiation therapy based on all-*trans* retinoic acid (RA) has dramatically improved patient outcomes for those suffering from acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML), but has been much less effective in treating other types of AML. This may be at least in part due to the heterogeneity of AML. A cell line used to model RA-induced differentiation, HL-60, is a non-APL AML, and our lab has recently shown that it may represent a previously uncharacterized subtype of AML. RA itself is known to regulate transcription of hundreds of genes, but the mechanistic details of the signaling pathways essential for differentiation are unclear. Here, we report interactions between the ectoenzyme receptor CD38 and the cytosolic signaling proteins Lyn, Vav1, SLP-76, and PI3K and show that Lyn also interacts with these cytosolic proteins. Building on prior results showing that Src family kinases enhance RA-induced differentiation, we explore the effects of several Src family kinase inhibitors on the process. We find that their effects on Lyn expression rather than their effects on Lyn activity appear to drive the process and that a clinically relevant inhibitor showing relatively low toxicity, bosutinib, behaves similarly to the more toxic inhibitor dasatinib. We also demonstrate that the ectoenzyme receptor CD38, which had a body of literature implicating it as a key driver of RA-induced differentiation, is not essential for the process. CXCR5, a receptor shown

to be essential for RA-induced differentiation, drives migration toward its ligand, CXCL13. While the mechanism by which CXCR5 contributes to the differentiation process remains unknown, we show that it does not appear to function via interaction with a number of candidate binding partners (CD11b, CD38, CXCR4, CXCR5, EBI2, c-Cbl, and Lyn). Finally, as histone deacetylases are known to be aberrantly active in various cancers, we show that a drug targeting the histone deacetylase Sirt1 does not affect differentiation, that two broadly targeting Sirt2 enhance differentiation concurrent with signs of toxicity, and that one specifically targeting Sirt2 is toxic but does not enhance differentiation. These findings contribute to the identification of targets to bring differentiation therapy to AML.

BIOGRAPHICAL SKETCH

Robert MacDonald graduated from Stoneham High School in 2007 and earned his combined Bachelor of Arts/Master of Arts degrees in biochemistry and molecular biology and biotechnology, respectively, at Boston University in 2011. While at Boston University, he gained his first experience performing research in Dr. Karen Allen's laboratory, working with Dr. Nicholas DeNunzio on the crystallization of truncated forms of botulinum neurotoxin. Wanting to combine his background with a dash of environmental science, he began Cornell University's Ph.D. program in environmental toxicology in 2011 in Dr. Andrew Yen's laboratory. During his time at Cornell University, he began to realize the importance of spreading science outside of the laboratory setting and was fortunate to have been accepted to attend a workshop on science communication, ComSciCon 14. He and another then-graduate student from Cornell, Dr. Kristin Hook, met there and co-founded ComSciCon Cornell in 2015, a workshop that has been held annually since its inception with the goal of providing STEM field graduate students and post-docs from upstate New York with a crash course in communicating complex topics to non-expert audiences. He ultimately hopes to use his education and experience to promote and uphold science's place in policymaking.

Dedicated to my father, Daniel, my mother, Constance, my sister, Sandra, and my girlfriend, Mikayla

ACKNOWLEDGMENTS

I am deeply grateful for my Principle Investigator and Committee Chair, Dr. Andrew Yen, whose support, guidance, and wisdom have been instrumental in shaping my research career at Cornell University. I also thank him for being available at any hour of the night to answer questions and provide feedback on my work.

I also thank the other members of my committee, Dr. Anthony Hay and Dr. Hening Lin, for their advice and insights on the various projects I have had over the years. I thank Dr. Hening Lin and members of his laboratory, particularly Dr. Jonathan Shrimp, Dr. Hong Jiang, Lu Zhang, and Hui Jing for collaborating with me on several projects. I also thank Dr. Anthony Hay for his tutelage as the instructor of several classes I have taken while at Cornell University, including one supremely personalized course in which I was his only pupil, and for always being willing to talk about our latest endeavors in distance running.

I thank my labmates for their constant support, recommendations, and collaborations. In particular, I thank Dr. Rodica Petruta Bunaciu, Dr. Johanna Congleton, Dr. Wendy Geil, and Dr. Holly Jensen. I also thank Victoria Ip and David Tran for their collaboration on one of our most recent projects. I thank David Dai, Dr. Jeffrey Varner, and my many other collaborators for their support. I thank Dorian LaTocha for his guidance in fluorescence-activated cell sorting and flow cytometric analysis. I also thank Dr. Robert Weiss for serving as my field-appointed member and for his work to establish connections between cancer researchers, patients, and survivors. I thank Susi Varvayanis for her tireless work as director of Cornell's Broadening Experiences in Scientific Training (BEST) program, which has provided me with countless opportunities for professional growth. I thank Dr. Kristin Hook, Dr. Susanna Kohler, and Susi Varvayanis for their collaboration and

guidance in starting ComSciCon Cornell, an annual science communication workshop serving graduate students and post-docs across upstate New York, and the ever-growing list of organizers who continue to make the event possible.

I also acknowledge the financial support I have received from Cornell University's Paracelcian Fellowship, the National Institutes of Health, the Cornell BEST program, and the Cornell Stem Cell program, without which my studies would not have been possible. I note that the content is solely the responsibility of the author and does not necessarily represent the official views of the National Institutes of Health.

I thank my friends and running partners for their companionship, encouragement, and dry humor on and off the track (or street, or path).

Finally, I thank my parents, Constance and Daniel MacDonald, my sister, Sandra MacDonald, and my girlfriend, Mikayla Scaduto, for their unwavering love and support.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1: Introduction

1.1 Cancer treatments – past, present, and future

Cancer is a uniquely heterogeneous disease with highly varied treatments. Although there has been tremendous progress in our understanding of the condition and treatment options have expanded significantly over the last several decades, cancer remains one of the leading causes of death worldwide.[1] Historically, these treatments have largely fallen into three categories: surgery, radiation therapy, and chemotherapy.

The premise of surgery to treat cancer, which dates back to ancient times, is focused on removal of malignant cells, generally present as localized tumors, from the body.[2] Radiation therapy was first explored as a way to diminish tumor size in 1896, shortly after its discovery.[3] The basis for its routine clinical use in the treatment of cancers, however, was not established until the mid-1930s, following the work of Henri Coutard.[4,5] Chemotherapy came about in the mid-1900s and involves the use of compounds that kill rapidly dividing cells.[6] Such agents affect stages of the cell growth and division process; for example, the DNA replication process is a common target of chemotherapeutic drugs, which alkylate or intercalate between DNA bases to induce mutations and ultimately cell death.[6,7]

While each of these treatments have proven effective when used alone and in combination with one another, they are by nature less than optimal treatments in their propensity to damage healthy as well as malignant cells, making the treatments and recovery processes difficult for patients. Surgery can involve significant trauma to surrounding tissue and is often limited to situations when cancer is present in a localized area. Radiation, similarly, is most effective in localized regions and may also damage

surrounding tissue. Chemotherapeutic drugs, which affect rapidly dividing cells, often kill cancer cells as well as healthy cells such as hair follicle cells, and perhaps more distressingly, those of the bone marrow and gastrointestinal tract.[8]

More selective treatments, which specifically target cancer cells while leaving properly functioning cells intact, will reduce the burden on the body. The primary mission of these treatments, known as targeted therapies, is to effectively eliminate or cripple cancer while mitigating side effects. There are many methods for doing so, all in various stages of development or clinical popularity, including hormone therapy and small molecule therapy.[9–12]

Several of these targeted therapies are also classified as immunotherapies, which are have perhaps generated the most interest among scientists and clinicians in recent years. Types of immunotherapies include antibody therapy, cancer vaccines, and chimeric antigen receptor (CAR) T-cell therapy. Antibody therapy involves the use of monoclonal antibodies. These may be conjugated with toxic drugs and target proteins overexpressed on cancer cells; they may be used as naked antibodies to promote or relieve inhibition of lymphocyte-mediated cancer cell death; or they may be bispecific, targeting proteins overexpressed in cancer and T-cells to create or boost an immune response against the cancer cells.[13] Cancer vaccines may activate the immune system against viruses that are correlated with cancer, such as human papilloma virus, or may be created on a personal level to generate an immune response against mutant proteins expressed by cancer cells.[14,15] CAR T-cell therapy, too, is highly personalized; a subset of a patient's own T cells are isolated, transduced to express a receptor against a specific protein expressed

on cancer cells, amplified, and then reintroduced into the patient to attack the cancer cells.[16,17]

While many immunotherapies have shown promising results, most are in early stages of development and few are currently approved by the Food and Drug Administration for clinical use. The subject of the studies in this dissertation is one of the oldest clinically relevant targeted therapies, differentiation therapy involving the use of all-*trans* retinoic acid (RA) to treat acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML).[18] Since the advent of RA in the 1980s and 1990s, APL patient outcome has dramatically improved, with remission rates going from under 60% to nearly 95%.[19,20] RA is highly effective but when used as a single agent, relapse frequently occurs within a matter of months.[20] Combination therapy with anthracyclines or, more recently, arsenic trioxide, has become the standard of care and relapse rates are minimal at only about 5-10%.[21] Unfortunately, RA has been far less effective in non-APL AML, where remission rates tend to fall between 50-70% at best.[22]

AML is a heterogeneous and relatively uncommon cancer, accounting for just over 20,000 of the nearly 1.7 million new cases of cancer diagnosed in the United States each year.[23,24] As a subtype of AML, APL accounts for about 10% of all AML cases.[25] APL is almost always characterized by a particular chromosomal translocation, t(15;17), that results in the fusion of promyelocytic leukemia protein (PML) and retinoic acid receptor α (RARα) to form PML-RARα.[25] A range of other chromosomal translocations, inversions, and deletions also occur in AML, affecting a variety of genes such as RUNX1, c-KIT, NPM1, FLT3, CEBPA, and IDH1/2.[24,26] Greater understanding of how subtypes with

particular genetic profiles respond to RA and other treatments may significantly improve patient outcome.

Our ultimate goal is to elucidate mechanistic details about RA-induced differentiation in the hopes that we may identify a key early protein or pathway seminal to the process. Once found, such targets may be actionable so that the efficacy of combination therapies based on RA could be improved in non-APL AML.

1.2 RA has various biological roles

RA is a metabolite of vitamin A and has roles in many physiological processes. It specifies the anterior/posterior axis of the developing embryos of chordate animals, acting through the Hox gene family.[27] RA helps to maintain epithelial surfaces of a variety of tissues, including the gut and respiratory tract, has functions in bone remodeling, and is used to treat many dermatological conditions.[28–31] RA also helps to regulate differentiation of hematopoietic stem cells and is critical for immune system development and function, where it is required for maturation of B cells.[32,33]

At physiological levels, RA induces granulocytic differentiation of hematopoietic stem cells; at higher levels, RA is able to drive the process in malignant cells in APL.[18] RA and one of its isomers, 9-cis retinol, accomplish this because they are ligands of the retinoic acid receptor (RAR) and retinoid X receptor (RXR).[34,35] They activate transcription of several hundred genes via RAR and RXR. These nuclear receptors can be found as homodimers or heterodimers and they each have three subtypes: alpha, beta, and gamma. RAR and RXR function as transcription factors and bind DNA at retinoic acid response element (RARE) sites present in hundreds of genes.[36] RAREs have AGGTCA hexad half-site consensus sequences and may occur in direct or inverted repeats (DR or

IR) separated by several nucleotides; RAR binds at sites separated by two or five nucleotides (DR2 or DR5).[37] Non-canonical RAREs, however, are also known to exist, necessitating experimental approaches to determine genes modulated by RA.[38]

The mechanism of action of RA in differentiation therapy involves de-repressing the DNA near RAREs. In the absence of their ligands, RAR and RXR dimers associate with repressor complexes that deacetylate nearby histones.[39] These repressor complexes are comprised of a histone deacetylase (HDAC) bound to nuclear co-repressor protein 2 (NCoR), also known as a silencing mediator for retinoid or thyroid-hormone receptors (SMRT), which is in turn bound to the RAR/RXR dimer.[40] The local transcriptional repression caused by the HDAC is relieved when a ligand binds to one or both of the RAR/RXR proteins that comprise the dimer. Under the ligand-bound condition, there is a disruption of the NCoR/SMRT binding domain and a co-activator complex is recruited to the dimer, consisting of proteins with histone acetyltransferase (HAT) activity such as p300 and CREB binding protein (CBP).[41] The HATs then acetylate the local histones, reversing the transcriptional repression. As described in the case of one RA-regulated gene, CXCR5, MAPK activity may also be required for transcription through phosphorylation of other regulatory transcription factors.[42] A simplified graphic is shown in Figure 1.1.

Apart from transcriptional modulation through RAR and RXR, RA is known to have many other cellular effects. RA can cause retinoylation, or retinoic acid acylation, of proteins, affecting fibroblast cell growth and biosynthesis of testosterone.[43–45] It can transrepress AP1-responsive genes by competing for Raf/MEK/ERK MAPK factors.[46] RA can also cause phosphorylation of transcription factors such as CREB, lead to both histone

acetylation and methylation events at various sites, and activate c-Raf via interaction with a hydrophobic pocket.[47–50]

Many models are used to study the effects and mechanism of action of RA, and in the context of non-APL AML, the HL-60 cell line is among the most common of these. HL-60 is a bipotent human myeloblastic leukemia cell line originally isolated in the 1970s from a patient with myeloblastic leukemia.[51,52] HL-60 can be induced to undergo granulocytic differentiation or monocytic differentiation in response to certain environmental cues and is classified under the French-American-British (FAB) hematologic system as M2.[52,53] Although it is responds to RA treatment like APL, HL-60 itself is not an APL, which are classified as FAB M3, and does not possess the translocation between chromosomes 15 and 17, or t(15,17), typically characteristic of the condition.[53] The translocation results in the formation of a fusion protein between RAR alpha and promyelocytic leukemia protein (PML), called PML-RAR alpha, which has a much higher affinity for some splice variants of NCoR/SMRT, leading to a stronger repression that can be overcome with pharmacological but not physiological levels of RA.[54] A recent publication from our lab demonstrated that the HL-60 cell line may be representative of a previously uncharacterized subtype of AML for which RA could be an effective therapy, making it an attractive model.[26]

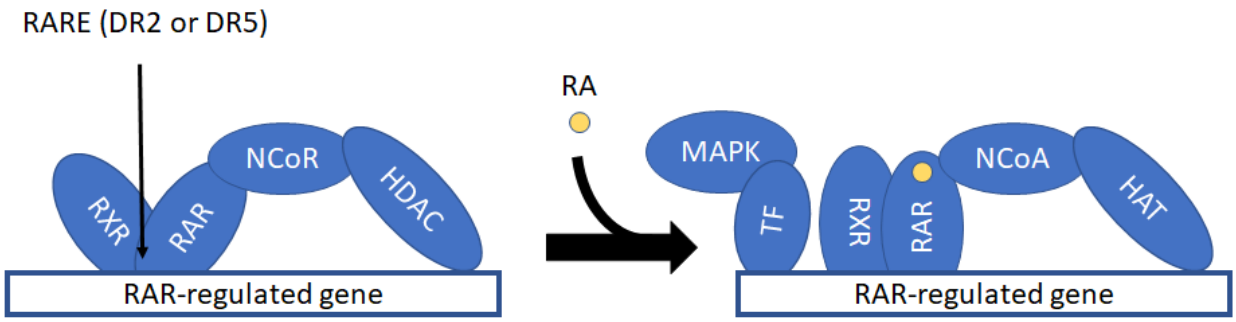


Figure 1.1: Simplified mechanism of RA-mediated transcriptional de-repression. RAR and RXR bind at RAREs of regulated genes and repress transcription through recruitment of HDACs via NCoRs. Following binding of RA to RAR, a conformational change occurs to allow recruitment of HATs through nuclear coactivators (NCoAs). HATs acetylate surrounding histones, allowing for transcription of the RAR-regulated gene. Other transcription factors (TFs) phosphorylated by MAPKs may also be required, as in the case of CXCR5, which requires NFATc3 phosphorylated by c-Raf in order to be transcribed.

1.3 MAPK signaling in RA-induced differentiation

Perhaps the most important signal generated as a result of RA treatment is a mitogen activated protein kinase (MAPK) cascade consisting of c-Raf, MEK1/2 (MEK), and ERK1/2 (ERK). This signal, unlike the classical short-lived MAPK signaling often generated by receptor tyrosine kinases, is prolonged, lasting for over 48 hours.[55] Durable MAPK signaling is associated with differentiation, whereas transient activation is associated with mitogenesis, as compared in the case of nerve versus epidermal growth factor.[56] Blocking the signaling cascade, accomplished via the MEK inhibitor PD98059 or the Raf inhibitor GW5074, cripples differentiation.[55,57] Conversely, ectopic expression of c-Raf accelerates RA-induced differentiation.[58] In addition to the unusual durable MAPK signal, a paradigm-shifting c-Raf nuclear translocation has been observed and is believed to be a key component of the differentiation process.[59] Classically, early c-Raf and MEK signaling is believed to propel ERK signaling and nuclear translocation to ultimately modulate gene expression. c-Raf phosphorylated at serine 621, however, is present in the nucleus upon RA-induced differentiation, where it binds NFATc3 at the promoter of CXCR5 and presumably other genes to activate transcription.[59,42] Both differentiation and its presence in the nucleus are crippled when PD98059 is used to disrupt MEK activity.[42] While the durable MAPK signal may be the ultimate driver of RA-induced differentiation in APL, we do not understand the mechanistic details of how the signal is first generated. c-Raf expression is increased in the process, but overexpression of c-Raf itself is not sufficient to induce differentiation on its own; the protein must be activated.[58] Two prime candidates that may be responsible for activation of c-Raf and thus the MAPK signal are the membrane-bound receptors CD38 and CXCR5.

1.4 Motivation: CD38 as a potential early driver of differentiation

CD38 is among the earliest proteins expressed following RA treatment, and it is also among the most dramatically upregulated, hinting at its potential significance in the process.[60,61] It is a type II transmembrane glycoprotein and its transcription is regulated by a RARE in the first intron, conveying its response to RA.[60] CD38 has both enzyme and receptor functions. Reports have demonstrated that although enzymatic activity of CD38, catalyzing the conversion of NAD^+ to cADPR and ADPR, is not important for RA-induced differentiation of HL-60 cells, CD38 does appear to play a role in the process.[62] Inhibition of CD38 by siRNA is reported to inhibit differentiation, while ectopic expression of CD38 levels comparable to those induced by RA enhances RA-induced differentiation.[61,63] Ectopic overexpression of CD38, however, cripples the process.[61] Interestingly, ERK activity thought essential for RA-induced differentiation is enhanced in both ectopic expressors and overexpressors when cultured in higher serum concentrations.[61]

The exact manner in which CD38 affects RA-induced differentiation remains an enigma, although there are indications that it does so through the previously mentioned MAPK cascade. CD38 is capable of activating two of its members, c-Raf and ERK.[64,65] CD38 can also interact with and induce phosphorylation of c-Cbl, an adaptor protein that can regulate MAPK signaling and is required for RA-induced differentiation.[66,67] CD38 is able to dimerize and tetramerize with itself, and inducing aggregation of CD38 with monoclonal antibodies generates a short-lived signal through c-Cbl.[64,68] Finally, CD38 has been shown to interact with other cell surface proteins in different contexts, such as a CD19/CD81 complex in lipid rafts in B cells; the interaction is necessary for CD38-mediated ERK signaling.[69] Interestingly, CD19 can act as an adapter, mediating receptor

signaling via Vav1, Src family kinases (SFKs), and PI3K; CD81 also is known to interact with other receptors and is capable of generating an ERK signal.[70–72]

Taken together, this evidence suggests the existence of a CD38-associated signaling complex capable of driving RA-induced differentiation and that it may depend on its functions as a receptor rather than an enzyme. Greater understanding of the receptor functions of CD38 and how they may be tied to RA-induced differentiation may yield valuable insight and reveal key early signaling events necessary for the process to occur.

1.5 Motivation: CXCR5 as a key galvanizer of RA-induced differentiation

CXCR5, originally discovered as Burkitt's Lymphoma Receptor 1 (BLR1), is best known for driving migration toward CXCL13 and is highly expressed in naïve B cells and T follicular helper cells, but it has also been implicated as a potential key driver of RA-induced differentiation.[58,73–77] CXCR5 is essential for the process, as bi-allelic knockout of the protein cripples RA-induced differentiation of HL-60 cells.[58] The protein was originally found in a subtractive hybridization screen between Burkitt's lymphoma patient cells to B cells immortalized using Epstein Barr virus, implicating it in the disease.[78] CXCR5 expression is driven by a non-canonical RARE, and mRNA expression is upregulated following RA treatment of HL-60 cells, although unlike with CD38, these levels are increased over longer time periods, peaking 48-72 h after RA treatment.[38,77]

CXCR5 is also reported to drive MAPK signaling along the c-Raf/MEK/ERK axis, activation of which drives RA-induced differentiation; ectopic expression of the protein results in enhancements of c-Raf S621, MEK, and ERK phosphorylation.[58,77] The mechanism by which CXCR5 is connected to this axis, however, is not known. It is

possible that CXCR5 is able to interact with other membrane receptors or downstream proteins in order to drive the MAPK signal and, in turn, cellular differentiation.

1.6 Motivation: SFK inhibitors in RA-induced differentiation

Several SFKs, Lyn, Hck, and Fgr, are overexpressed and abnormally activated in AML cells.[79] SFKs are often found near the plasma membrane, where they contribute to cancer progression and survival through regulation of proliferation, migration, and adhesion, making them an attractive target for therapeutic intervention.[80–83] They are also known to positively regulate MAPK signaling and contribute to cell transformation.[84] Lyn is of particular interest; high expression levels are associated with more favorable cytogenetic risk groups, indicating higher likelihood of long-term survival, and it is the predominantly active SFK in HL-60 cells.[79,85,86]

The SFK inhibitors PP2 and dasatinib enhance RA-induced differentiation of HL-60 cells.[87] PP2, however, is not a specific inhibitor of SFKs and broadly inhibits tyrosine kinases.[88] Dasatinib, too, is broad inhibitor of tyrosine kinases, though it is currently in use for treating chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL).[89–91] Many patients, however, cannot tolerate dasatinib due to its toxicity.[92,93] These inhibitors are known to have positive effects on activation of c-Raf through its S259 and S621 phosphorylation sites and may thus drive signaling along the c-Raf/MEK/ERK axis, promoting RA-induced differentiation.[87] A milder SFK inhibitor capable of activating MAPK signaling may therefore be an attractive candidate for combination therapy with RA in AML.

1.7 Motivation: Histone deacetylase (HDAC) inhibitors in RA-induced differentiation

HDAC inhibitors represent another class of drugs that may be useful for the treatment of AML in combination with RA. HDACs are a class of proteins that help to regulate gene expression by altering DNA packaging; histone acetylation is reported to weaken attractive forces between negatively charged DNA and positively charged lysine resides of histones.[94] In particular, HDACs and their counterparts, histone acetyl transferases, are known to regulate transcription of RARs.[39] While histone acetylation is generally thought of as a feature of actively transcribed genes, as in the case of histone 3 lysine 9, 14, and 27 acetylation, this modification is not a universal indicator of such activity.[95] Histone 4 lysine 20 acetylation, for example, is a mark associated with gene repression.[96] Given their role in transcriptional regulation, it is unsurprising that HDACs are known to exhibit aberrant activity in a variety of cancers.[97,98] Many HDAC inhibitors have been shown to induce apoptosis in a variety of cancers.[98,99] There are also reports that some HDAC inhibitors, such as valproic acid, and sodium butyrate, are able to induce differentiation or enhance RA-induced differentiation of HL-60 cells.[100–102] These, however, are promiscuous inhibitors that affect a range of HDACs.[103,104] As the proteins' activity is also essential for normal regulation of gene transcription, compounds that target specific HDACs may present more attractive options for study and potential clinical use.

In AML, the class III HDAC and member of the sirtuin family, Sirt2, is particularly interesting. Sirt2 has been shown to mediate expression of multidrug resistance-associated protein 1 (MDR1), which is correlated with AML prognosis.[105,106] Higher levels of Sirt2 are seen in relapsed AML patients, and high Sirt2 levels are also indicative of poor prognosis.[106,107] Depletion of Sirt2 reduces MDR1 levels, suggesting that therapeutic intervention targeting Sirt2 may improve patient outcomes.[106]

1.8 Project overview

Here, we assessed several aspects of RA-induced differentiation in the HL-60 cell line in pursuit of mechanistic details of how RA-induced differentiation works and whether certain other drugs may make attractive candidates for co-treatment with RA. This report details the use of two different CD38 linkers, the monoclonal antibody IB4 and a dimeric version of the suicide substrate, dimeric F-araNAD$^+$, as well as several CRISPR-mediated CD38 truncations, in order to probe how CD38 is tied to downstream MAPK signaling and whether it is a key early driver of RA-induced differentiation. We searched for CXCR5 interaction partners to determine whether dimerization with any of a set of candidates may offer insight as to how it is able to drive MAPK signaling. We also characterized the effects of a Src family kinase inhibitor known for its relatively benign toxicity profile, bosutinib, on RA-induced differentiation. Finally, we tested several inhibitors of members of the sirtuin family of HDACs, a specific Sirt1 inhibitor, two less specific Sirt2 inhibitors, as well as recently developed specific Sirt2 inhibitor, to determine their effects on RA-induced differentiation.

REFERENCES

1. Wang, H. *et al.* Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: A systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388,** 1459–1544 (2016).
2. Sudhakar, A. History of cancer, ancient and modern treatment methods. *J. Cancer Sci. Ther.* **1,** i–iv (2009).
3. Cosset, J.-M. The dawn of radiotherapy, between strokes of genius, dramas and controversies. *Cancer / Radiother.* **20,** 595–600 (2016).
4. Webster, J. H. D. The protracted-fractional x-ray method (coutard) in the treatment of cancer of the larynx. *J. Laryngol. Otol.* **49,** 429–449 (1934).
5. Young, B. R. Should the method of Coutard be applied in all cases of cancer treated by Roentgen rays? *Radiology* 186–189 (1937).
6. DeVita, V. T. & Chu, E. A history of cancer chemotherapy. *Cancer Res.* **68,** 8643–8653 (2008).
7. Malhotra, V. & Perry, M. C. Classical chemotherapy: mechanisms, toxicities and the therapeutic window. *Cancer Biol. Ther.* **2,** S2-S4 (2003).
8. Raji, M. A. Management of chemotherapy-induced side-effects. *Lancet Oncol.* **6,** 357 (2005).
9. Brawer, M. K. Hormonal therapy for prostate cancer. *Rev. Urol.* **8 Suppl 2,** S35-S47 (2006).
10. Jordan, V. C. Tamoxifen: Catalyst for the change to targeted therapy. *Eur. J. Cancer* **44,** 30–38 (2008).
11. Warr, M. R. & Shore, G. C. Small-molecule Bcl-2 antagonists as targeted therapy in oncology. *Curr. Oncol.* **15,** 2–7 (2008).
12. Katzel, J. A., Fanucchi, M. P. & Li, Z. Recent advances of novel targeted therapy in non-small cell lung cancer. *J. Hematol. Oncol.* **2,** 2 (2009).
13. Scott, A. M., Wolchok, J. D. & Old, L. J. Antibody therapy of cancer. *Nat. Rev. Cancer* **12,** 278–287 (2012).
14. Sahin, U. & Türeci, Ö. Personalized vaccines for cancer immunotherapy. **1360,** 1355–1360 (2018).
15. Banchereau, J. & Palucka, K. Immunotherapy: Cancer vaccines on the move. *Nat. Rev. Clin. Oncol.* **15,** 9–10 (2017).
16. Gross, G., Waks, T. & Eshhar, Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 10024–10028 (1989).
17. Smith, A. J., Oertle, J., Warren, D. & Prato, D. Chimeric antigen receptor (CAR) T cell therapy for malignant cancers: Summary and perspective. *J. Cell. Immunother.* **2,** 59–68 (2016).
18. Warrell, R. P. *et al.* Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N. Engl. J. Med.* **324,** 1385–1393 (1991).
19. Bernard, J. *et al.* Acute promyelocytic leukemia: Results of treatment by daunorubicin. *Blood* **41,** 489–496 (1973).
20. Wang, Z. Y. & Chen, Z. Acute promyelocytic leukemia: From highly fatal to highly curable. *Blood* **111,** 2505–2515 (2008).

21. Coombs, C. C., Tavakkoli, M. & Tallman, M. S. Acute promyelocytic leukemia: Where did we start, where are we now, and the future. *Blood Cancer J.* **5,** e304 (2015).
22. Ma, H. S., Robinson, T. M. & Small, D. Potential role for all- trans retinoic acid in nonpromyelocytic acute myeloid leukemia. *Int. J. Hem. Onc.* **5,** 133–142 (2017).
23. Understanding Cancer: Cancer Statistics. *National Cancer Institute* (2018). Available at: https://www.cancer.gov/about-cancer/understanding/statistics. (Accessed: 24th April 2018)
24. De Kouchkovsky, I. & Abdul-Hay, M. Acute myeloid leukemia: A comprehensive review and 2016 update. *Blood Cancer J.* **6,** e441 (2016).
25. De Braekeleer, E., Douet-Guilbert, N. & De Braekeleer, M. *RARA* fusion genes in acute promyelocytic leukemia: A review. *Expert Rev. Hematol.* **7,** 347–357 (2014).
26. Bunaciu, R. P. *et al.* Potential for subsets of wt-NPM1 primary AML blasts to respond to retinoic acid treatment. *Oncotarget* **5,** 4134–4149 (2017).
27. Shimeld, S. M. Retinoic acid, HOX genes and the anterior-posterior axis in chordates. *BioEssays* **18,** 613–616 (1996).
28. Holland, R. E., Pfeiffer, C. J., Bruns, N. J. & Webb, K. E. Morphologic alterations in small intestinal epithelium of lambs fed vitamin A-depleted diet. *Dig. Dis. Sci.* **38,** 333–343 (1993).
29. Million, K. *et al.* Effects of retinoic acid receptor-selective agonists on human nasal epithelial cell differentiation. *Am. J. Respir. Cell Mol. Biol.* **25,** 744–750 (2001).
30. Togari, A., Kondo, M., Arai, M. & Matsumoto, S. Effects of retinoic acid on bone formation and resorption in cultured mouse calvaria. *Gen. Pharmacol.* **22,** 287–292 (1991).
31. Rolewski, S. L. Clinical review: Topical retinoids. *Dermatology Nurs.* **15,** 447-450 (2003).
32. Purton, L. E., Bernstein, I. D. & Collins, S. J. All-trans retinoic acid delays the differentiation of primitive hematopoietic precursors (lin-c-kit+Sca-1(+)) while enhancing the terminal maturation of committed granulocyte/monocyte progenitors. *Blood* **94,** 483–495 (1999).
33. Ross, A. C., Chen, Q. & Ma, Y. Vitamin A and retinoic acid in the regulation of B-cell development and antibody production. *Vitam. Horm.* **86,** 103-126 (2011).
34. Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. & Evans, R. M. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345,** 224–229 (1990).
35. Mangelsdorf, D. J. *et al.* Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6,** 329–344 (1992).
36. Balmer, J. E. & Blomhoff, R. Gene expression regulation by retinoic acid. *J. Lipid Res.* **43,** 1773–1808 (2002).
37. Mangelsdorf, D. J. & Evans, R. M. The RXR heterodimers and orphan receptors. *Cell* **83,** 841–850 (1995).
38. Wang, J. & Yen, A. A novel retinoic acid-responsive element regulates retinoic acid-induced BLR1 expression. *Mol. Cell. Biol.* **24,** 2423–2443 (2004).
39. Altucci, L. & Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat. Rev. Cancer* **1,** 181–193 (2001).
40. Lazar, M. Nuclear receptor corepressors. *Nucl. Recept. Signal.* **1,** e001 (2003).
41. Chekravarti, D. *et al.* Role of CBP/P300 in nuclear receptor signalling. *Nature* **383,**

99–103 (1996).
42. Geil, W. M. & Yen, A. Nuclear Raf-1 kinase regulates the CXCR5 promoter by associating with NFATc3 to drive retinoic acid-induced leukemic cell differentiation. *FEBS J.* **281,** 1170–1180 (2014).
43. Takahashi, N. & Breitman, T. R. Retinoic acid acylation (retinoylation) of a nuclear protein in the human acute myeloid leukemia cell line HL60. *J. Biol. Chem.* **264,** 5159–5163 (1989).
44. Lacroix, A. & Lippman, M. E. Binding of retinoids to human breast cancer cell lines and their effects on cell growth. *J. Clin. Invest.* **65,** 586–591 (1980).
45. Cione, E., Pingitore, A., Perri, M. & Genchi, G. Influence of all-trans-retinoic acid on oxoglutarate carrier via retinoylation reaction. *Biochim. Biophys. Acta.* **1791,** 3–7 (2009).
46. Dedieu, S. & Lefebvre, P. Retinoids interfere with the AP1 signalling pathway in human breast cancer cells. *Cell. Signal.* **18,** 889–898 (2006).
47. Cañón, E., Cosgaya, J., S, S. & Aranda, A. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. *Mol. Biol. Cell* **15,** 5583–5592 (2004).
48. Kashyap, V. & Gudas, L. J. Epigenetic regulatory mechanisms distinguish retinoic acid-mediated transcriptional responses in stem cells and fibroblasts. *J. Biol. Chem.* **285,** 14534–14548 (2010).
49. Hoyos, B. *et al.* Activation of c-Raf kinase by ultraviolet light: Regulation by retinoids. *J. Biol. Chem.* **277,** 23949–23957 (2002).
50. Hoyos, B., Jiang, S. & Hammerling, U. Location and functional significance of retinol-binding sites on the serine/threonine kinase, c-Raf. *J. Biol. Chem.* **280,** 6872–6878 (2005).
51. Collins, S. J., Gallo, R. C. & Gallagher, R. E. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* **270,** 347–349 (1977).
52. Fontana, J. A., Colbert, D. A. & Deisseroth, A. B. Identification of a population of bipotent stem cells in the HL60 human promyelocytic leukemia cell line. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3863–3866 (1981).
53. Dalton, W. T. *et al.* HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood* **71,** 242–247 (1988).
54. Mengeling, B. J., Phan, T. Q., Goodson, M. L. & Privalsky, M. L. Aberrant corepressor interactions implicated in PML-RAR(alpha) and PLZF-RAR(alpha) leukemogenesis reflect an altered recruitment and release of specific NCoR and SMRT splice variants. *J. Biol. Chem.* **286,** 4236–4247 (2011).
55. Yen, A., Roberson, M. S., Varvayanis, S. & Lee, A. T. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res.* **58,** 3163–3172 (1998).
56. Traverse, S., Gomez, N., Paterson, H., Marshall, C. & Cohen, P. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem. J.* **288 (Pt 2,** 351–5 (1992).
57. Reiterer, G. *et al.* RAF associates with phosphorylated nuclear BubR1 during endoreduplication induced by JAK inhibition. *Cell Cycle* **9,** 3297–3304 (2010).

58. Wang, J. & Yen, A. A MAPK-positive feedback mechanism for BLR1 signaling propels retinoic acid-triggered differentiation and cell cycle arrest. *J. Biol. Chem.* **283,** 4375–4386 (2008).
59. Smith, J. *et al.* Retinoic acid induces nuclear accumulation of Raf1 during differentiation of HL-60 cells. *Exp. Cell Res.* **315,** 2241–2248 (2009).
60. Kishimoto, H. *et al.* Molecular mechanism of human CD38 gene expression by retinoic acid. Identification of retinoic acid response element in the first intron. *J. Biol. Chem.* **273,** 15429–15434 (1998).
61. Lamkin, T. J. *et al.* Retinoic acid-induced CD38 expression in HL-60 myeloblastic leukemia cells regulates cell differentiation or viability depending on expression levels. *J. Cell. Biochem.* **97,** 1328–1338 (2006).
62. Congleton, J., Jiang, H., Malavasi, F., Lin, H. & Yen, A. ATRA-induced HL-60 myeloid leukemia cell differentiation depends on the CD38 cytosolic tail needed for membrane localization, but CD38 enzymatic activity is unnecessary. *Exp. Cell Res.* **317,** 910–919 (2011).
63. Munshi, C. B., Graeff, R. & Lee, H. C. Evidence for a causal role of CD38 expression in granulocytic differentiation of human HL-60 cells. *J. Biol. Chem.* **277,** 49453–49458 (2002).
64. Zubiaur, M., Izquierdo, M., Terhorst, C., Malavasi, F. & Sancho, J. CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-ζ/ζ-associated protein-70 signaling pathways in Jurkat T lymphocytes. *J. Immunol.* **159,** 193–205 (1997).
65. Zubiaur, M. *et al.* CD38 is associated with lipid rafts and upon receptor stimulation leads to Akt/protein kinase B and Erk activation in the absence of the CD3-ζ immune receptor tyrosine-based activation motifs. *J. Biol. Chem.* **277,** 13–22 (2002).
66. Kontani, K. *et al.* Tyrosine phosphorylation of the c-cbl proto-oncogene product mediated by cell surface antigen CD38 in HL-60 cells. *J. Biol. Chem.* **271,** 1534–1537 (1996).
67. Shen, M. & Yen, A. c-Cbl interacts with CD38 and promotes retinoic acid-induced differentiation and G0 arrest of human myeloblastic leukemia cells. *Cancer Res.* **68,** 8761–8769 (2008).
68. Hara-Yokoyama, M. *et al.* Tetrameric interaction of the ectoenzyme CD38 on the cell surface enables its catalytic and raft-association activities. *Structure* **20,** 1585–1595 (2012).
69. Zumaquero, E. *et al.* Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signaling complexes containing CD81, Hsc-70 and Lyn. *Exp. Cell Res.* **316,** 2692–2706 (2010).
70. Carter, R. H., Wang, Y. & Brooks, S. Role of CD19 signal transduction in B cell biology. *Immunol. Res.* **26,** 45–54 (2002).
71. Claas, C., Stipp, C. S. & Hemler, M. E. Evaluation of prototype transmembrane 4 superfamily protein complexes and their relation to lipid rafts. *J. Biol. Chem.* **276,** 7974–7984 (2001).
72. Carloni, V., Mazzocca, A. & Ravichandran, K. S. Tetraspanin CD81 is linked to ERK/MAPKinase signaling by Shc in liver tumor cells. *Oncogene* **23,** 1566–1574 (2004).

73. Förster, R. *et al.* A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* **87,** 1037–1047 (1996).
74. Legler, D. F. *et al.* B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J. Exp. Med.* **187,** 655–660 (1998).
75. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* **192,** 1545–1552 (2000).
76. Schaerli, P. *et al.* CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J. Exp. Med.* **192,** 1553–1562 (2000).
77. Battle, T. E., Levine, R. A. & Yen, A. Retinoic acid-induced blr1 expression promotes ERK2 activation and cell differentiation in HL-60 cells. *Exp. Cell Res.* **254,** 287–298 (2000).
78. Dobner, T., Wolf, I., Emrich, T. & Lipp, M. Differentiation-specific expression of a novel G protein-coupled receptor from Burkitt's lymphoma. *Eur. J. Immunol.* **22,** 2795–2799 (1992).
79. Dos Santos, C. *et al.* A critical role for Lyn in acute myeloid leukemia. *Blood* **111,** 2269–2279 (2008).
80. Thomas, S. M. & Brugge, J. S. Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* **13,** 513–609 (1997).
81. Wheeler, D. L., Iida, M. & Dunn, E. F. The role of Src in solid tumors. *Oncologist* **14,** 667–678 (2009).
82. Mayer, E. L. & Krop, I. E. Advances in targeting Src in the treatment of breast cancer and other solid malignancies. *Clin. Cancer Res.* **16,** 3526–3532 (2010).
83. Amrein, P. C. The potential for dasatinib in treating chronic lymphocytic leukemia, acute myeloid leukemia, and myeloproliferative neoplasms. *Leuk. Lymphoma* **52,** 754–763 (2011).
84. Kim, M. P., Park, S. I., Kopetz, S. & Gallick, G. E. Src family kinases as mediators of endothelial permeability: Effects on inflammation and metastasis. *Cell Tissue Res.* **335,** 249–259 (2009).
85. Iriyama, N., Yuan, B., Hatta, Y., Takagi, N. & Takei, M. Lyn, a tyrosine kinase closely linked to the differentiation status of primary acute myeloid leukemia blasts, associates with negative regulation of all-trans retinoic acid (ATRA) and dihydroxyvitamin D3 (VD3)-induced HL-60 cells differentiation. *Cancer Cell Int.* **16,** (2016).
86. Kropf, P. L., Wang, L., Zang, Y., Redner, R. L. & Johnson, D. E. Dasatinib promotes ATRA-induced differentiation of AML cells. *Leukemia* **24,** 663–665 (2010).
87. Congleton, J., MacDonald, R. & Yen, A. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. *Leukemia* **26,** 1180–1188 (2012).
88. Brandvold, K. R., Steffey, M. E., Fox, C. C. & Soellner, M. B. Development of a highly selective c-Src kinase inhibitor. *ACS Chem. Biol.* **7,** 1393–1398 (2012).
89. Davis, M. I. *et al.* Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* **29,** 1046–1051 (2011).

90. Keskin, D., Sadri, S. & Eskazan, A. E. Dasatinib for the treatment of chronic myeloid leukemia: Patient selection and special considerations. *Drug Des. Devel. Ther.* **10,** 3355–3361 (2016).
91. Foà, R. *et al.* Dasatinib as first-line treatment for adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* **118,** 6521–6528 (2011).
92. Kantarjian, H. M. *et al.* Dasatinib or imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: 2-Year follow-up from a randomized phase 3 trial (DASISION). *Blood* **119,** 1123–1129 (2012).
93. Kantarjian, H. M. *et al.* Bosutinib safety and management of toxicity in leukemia patients with resistance or intolerance to imatinib and other tyrosine kinase inhibitors. *Blood* **123,** 1309–1318 (2014).
94. Bannister, A. J. & Kouzarides, T. Regulation of chromatin by histone modifications. *Cell Res.* **21,** 381–395 (2011).
95. Wang, Z. *et al.* Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat. Genet.* **40,** 897–903 (2008).
96. Kaimori, J. Y. *et al.* Histone H4 lysine 20 acetylation is associated with gene repression in human cells. *Sci. Rep.* **6,** 24318 (2016).
97. Ropero, S. & Esteller, M. The role of histone deacetylases (HDACs) in human cancer. *Mol. Oncol.* **1,** 19–25 (2007).
98. Li, Y. & Seto, E. HDACs and HDAC inhibitors in cancer development and therapy. *Cold Spring Harb. Perspect. Med.* **6,** (2016).
99. West, A. C. & Johnstone, R. W. New and emerging HDAC inhibitors for cancer treatment. *J. Clin. Invest.* **124,** 30–39 (2014).
100. Deubzer, H. *et al.* Novel valproic acid derivatives with potent differentiation-inducing activity in myeloid leukemia cells. *Leuk. Res.* **30,** 1167–1175 (2006).
101. Bunaciu, R. P. & Yen, A. Activation of the aryl hydrocarbon receptor ahr promotes retinoic acid-induced differentiation of myeloblastic leukemia cells by restricting expression of the stem cell transcription factor Oct4. *Cancer Res.* **71,** 2371–2380 (2011).
102. Yen, A. & Varvayanis, S. DMSO, sodium butyrate, and TPA induce hypophosphorylation of RB with HL-60 cell differentiation. *In Vitro Dev. Biol. Anim.* **31,** 164–167 (1995).
103. Göttlicher, M. *et al.* Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* **20,** 6969–6978 (2001).
104. Chen, J. S., Faller, D. V & Spanjaard, R. a. Short-chain fatty acid inhibitors of histone deacetylases: promising anticancer therapeutics? *Curr. Cancer Drug Targets* **3,** 219–236 (2003).
105. Legrand, O., Zittoun, R. & Marie, J. P. Role of MRP1 in multidrug resistance in acute myeloid leukemia. *Leukemia* **13,** 578–584 (1999).
106. Xu, H. *et al.* SIRT2 mediates multidrug resistance in acute myelogenous leukemia cells via ERK1/2 signaling pathway. *Int. J. Oncol.* 613–623 (2015).
107. Deng, A., Ning, Q., Zhou, L. & Liang, Y. SIRT2 is an unfavorable prognostic biomarker in patients with acute myeloid leukemia. *Sci. Rep.* **6,** 27694 (2016).

Chapter 2 Foreword

The work in this chapter was motivated by our interest in both CD38 and SFK inhibitors. Earlier data on CD38 interaction partners generated by Dr. Miaoqing Shen (Figures 2.1 and 2.2) was paired with work probing CD38 ligation in combination with early and late SFK inhibitor treatment initiated by Dr. Johanna Congleton (parts of Figures 2.3-2.5) and completed by Robert MacDonald (parts of Figures 2.3-2.5). An initial draft was written by Dr. Johanna Congleton and edited by herself, Dr. Andrew Yen, and Robert MacDonald. Following a rejection, additional data was added to Figures 2.3 and 2.5 and the paper was re-written by Robert MacDonald, with edits provided by Dr. Johanna Congleton, Dr. Fabio Malavasi, and Dr. Andrew Yen. The paper was then accepted, and the full citation is below:

Congleton, J., Shen, M., MacDonald, R., Malavasi, F. & Yen, A. Phosphorylation of c-Cbl and p85 PI3K driven by all-trans retinoic acid and CD38 depends on Lyn kinase activity. *Cell Signal.* **7,** 1589-1597 (2014).

Chapter 2: Phosphorylation of c-Cbl and p85 PI3K driven by all-*trans* retinoic acid and CD38 depends on Lyn kinase activity

2.1 Abstract

The leukocyte antigen CD38 is expressed after all-*trans* retinoic acid (RA) treatment in HL-60 myelogenous leukemia cells and promotes induced myeloid differentiation when overexpressed. We found that Vav1 and SLP-76 associate with CD38 in two cell lines, and that these proteins complex with Lyn, a Src family kinase (SFK) upregulated by RA. SFK inhibitors PP2 and dasatinib, which enhance RA-induced differentiation, were used to evaluate the involvement of Lyn kinase activity in CD38-driven signaling. Cells treated with RA for 48 hours followed by one hour of PP2 incubation show SFK/Lyn kinase inhibition. We observed that Lyn inhibition blocked c-Cbl and p85/p55 PI3K phosphorylation driven by the anti-CD38 agonistic mAb IB4 in RA-treated HL-60 cells and untreated CD38+ transfectants. In contrast, cells cultured for 48 hours following concurrent RA and PP2 treatment did not show Lyn inhibition, suggesting RA regulates the effects on Lyn. 48 hours of co-treatment preserved CD38-stimulated c-Cbl and p85/p55 PI3K phosphorylation indicating Lyn kinase activity is necessary for these events. In contrast another SFK inhibitor (dasatinib) which blocks Lyn activity with RA co-treatment prevented RA-induced c-Cbl phosphorylation and crippled p85 PI3K phosphorylation, indicating Lyn kinase activity is important for RA-propelled events potentially regulated by CD38. We found that loss of Lyn activity coincided with a decrease in Vav1/Lyn/CD38 and SLP-76/Lyn/CD38 interaction, suggesting these molecules form a complex that regulates CD38 signaling. Lyn inhibition also reduced Lyn and CD38 binding to p85 PI3K, indicating CD38 facilitates a complex responsible for PI3K

phosphorylation. Therefore, Lyn kinase activity is important for CD38-associated signaling that may drive RA-induced differentiation.

2.2 Introduction

All-*trans* retinoic acid (RA) is used clinically to treat acute promyelocytic leukemia (APL), but is largely unsuccessful in treating other types of leukemias that are t(15,17) negative. HL-60 is a human acute myelogenous leukemia (AML) cell line that is t(15,17) negative and used as a model to study the mechanisms of RA-propelled myeloid differentiation in non-APL cells. Molecules and signaling pathways that confer RA responsiveness in HL-60 cells may be important in elucidating how a non-APL leukemia cell can be induced to differentiate by RA and may ultimately provide knowledge that could expand the use of RA as a therapeutic agent.

CD38 is a leukocyte antigen that is an early marker of RA induction whose expression is mediated via retinoic acid receptor α (RARα) and drives differentiation when overexpressed.[1,2] CD38 is an ectoenzyme receptor and has enzymatic activity that generates the Ca^{2+} mobilizing compounds NAADP+ and cADPR. It also has receptor functions that drive cell signaling including the phosphorylation of c-Cbl, extracellular signal-regulated kinase (ERK), and the p85 PI3K regulatory subunit.[2–9]

Enzymatic activity and receptor/signaling functions can operate independently.[10–12] For example, CD38 metabolic activity is unnecessary for RA-induced differentiation while the receptor function associated with membrane-expressed CD38 is required.[13] In addition, siRNA targeting CD38 cripples differentiation.[14] These reports suggest that CD38-driven signaling is important for RA-driven myeloid maturation. Therefore, it is of interest to

identify CD38-associated signaling molecules and how they may regulate RA efficacy. Such knowledge may indicate targets for therapeutic intervention.

CD38 forms a complex with c-Cbl and CD38 agonist ligand interaction results in c-Cbl phosphorylation.[3,15,16] c-Cbl is an E3 ubiquitin ligase and adaptor molecule that, like CD38, promotes mitogen-activated protein kinase (MAPK) signaling and RA-induced differentiation when overexpressed.[3,15,16] This suggests that the c-Cbl/CD38 interaction may cooperatively drive MAPK signaling and other aspects of RA therapy. This is consistent with a report that a c-Cbl tyrosine kinase binding domain mutant (G306E) that does not bind CD38 also fails to drive MAPK signaling and differentiation.[16]

c-Cbl is known to interact with the guanine nucleotide exchange factor Vav1, the SLP-76 adaptor, and, like CD38, the p85 regulatory subunit of PI3K.[15–18] c-Cbl, SLP-76, and Vav1 protein expression and p85 PI3K activity are upregulated during granulocytic maturation.[19–23] These four proteins also form complexes in myeloid cells after RA treatment. For example, Vav1 associates with PI3K and may facilitate the characteristic nucleoskeleton remodeling that occurs with RA treatment in HL-60 and NB4 cells.[24,25] Consistent with this, downmodulation of Vav1 impedes induced myeloid maturation and nucleoskeleton remodeling, and affects differentiation-related protein expression.[23] This suggests Vav1 may be a key regulator of myeloid differentiation.

The Src homology 2 domain of Vav1 interacts with c-Cbl and SLP-76 in a differentiation-dependent manner. After RA treatment Vav1/c-Cbl complexes are detectable in the cytosol, while Vav1/SLP-76 interactions are predominant in nuclei.[24] SLP-76 is also upregulated after RA and forms a complex with c-Cbl.[16] Co-expression of SLP-76 with

CSF-1/c-FMS enhances RA-induced ERK activation, G0 cell cycle arrest, and a number of additional differentiation markers.[21]

CD38 ligation by the anti-CD38 agonistic mAbs IB4 (IB4) and T16 induces phosphorylation of the p85 regulatory subunit of PI3K and is associated with normal and leukemic B cell growth suppression.[26–28] Consistent with this, PI3K inhibitors relieved CD38-mediated growth suppression in RA-treated HL-60 cells, which suggests a PI3K-modulated CD38 feedback loop.[29]

CD38 also drives transient MAPK activation after agonist ligation, which is orchestrated by the Raf/MEK/ERK axis.[6,7] Transient or protracted signaling from this cascade can lead to either cell proliferation or differentiation respectively, and sustained MAPK signaling characterizes RA-induced differentiation.[30–32] CD38 overexpression results in persistent ERK phosphorylation, therefore CD38 appears capable of propagating a transient or sustained MAPK signal.

Lyn and other Src family kinases (SFKs) are known to be modulated by RA treatment, and Lyn is linked to CD38-driven signaling events. For example, CD38 stimulation of B lymphocytes obtained from Lyn-deficient mice showed defective differentiation, and drugs interfering with PI3K or ERK decreased differentiation.[33] This suggests that Lyn may cooperate with other CD38-associated signaling molecules, such as PI3K and ERK. Other reports show that both Fyn and Lyn are required for B cell signaling after CD38 ligation.[34] Likewise, in lymphoblastoid B-cell membrane rafts CD38 is associated with Lyn and modulates cell signaling.[35]

SFK inhibitors are known to enhance aspects of RA induction, including expression of CD11b and other myeloid maturation markers.[36,37] A recent study reported that dasatinib,

which inhibits Lyn kinase activity alone and with RA co-adminstration, enhances differentiation.[38] However the inhibitor PP2, which inhibits Lyn alone but does not block kinase activity with RA co-treatment, shows a more significant enhancement of RA-induced differentiation than dasatinib. Therefore, Lyn kinase activity may function to drive some aspects of differentiation.

While neither inhibitor is specific for Lyn, the protein was the primary target of significance at the treatment levels used. PP2 can affect ZAP70 and JAK2, for example, but at higher concentrations; we found negligible expression of SFKs Fyn and Lck in HL-60s, and a recent study revealed that the SFK Fgr, while expressed in response to RA treatment, was not activated in response to RA, PP2, dasatinib, or RA and SFK inhibitor co-treatment.[38,39] HL-60s also do not express the BCR/ABL fusion protein, a target of dasatinib.

Since membrane-expressed CD38 has a role in differentiation, signaling that may involve c-Cbl, SLP-76, Vav1, PI3K, and Lyn are important in understanding how RA provides therapeutic benefit. Clarification of pathways that confer RA responsiveness in t(15,17) negative HL-60 cells could lead to new treatment targets in a larger array of leukemias, as well as other types of cancers. For example, RA has shown some promise in treating reproductive leiomyomas by modulating the PI3K signaling cascade.[40]

Our results show that CD38, SLP-76, and Vav1 were able to interact together in two cell lines, HL-60 and NB4. Lyn also complexed with these molecules and we evaluated if Lyn kinase activity had an effect on CD38 ligand-induced signaling, including phosphorylation of c-Cbl, ERK, and p85 PI3K. Using the SFK inhibitor PP2 we found that blocking Lyn kinase activity had modest effects on ERK phosphorylation, but was able to completely

abrogate c-Cbl and p85 PI3K phosphorylation driven by IB4.[28,41] We used CD38+ stable transfectants and RA-treated HL-60 cells to evaluate if the effects of the inhibitor were associated with either RA or CD38 expression alone, and found that PP2 blocked pY-c-Cbl and pY-p85 PI3K in both cell lines. A previous report showed that co-treatment with RA and PP2 followed by 48 hours of culture protects Lyn kinase activity from PP2 inhibition and significantly enhances differentiation.[38] Protecting Lyn kinase activity using RA/PP2 co-treatment also permitted CD38 ligand-induced phosphorylation of c-Cbl and p85 PI3K. We also observed that dasatinib, which unlike PP2 blocks Lyn activity with RA co-treatment, prevented RA-propelled c-Cbl phosphorylation and crippled p-p85 PI3K. Therefore, Lyn activity appears needed for two signaling events that are downstream of CD38 and induced by retinoic acid.

Finally, we observed that inhibition of Lyn decreased interactions among Vav1/Lyn/CD38, SLP-76/Lyn/CD38, and p85 PI3K/Lyn/CD38. Importantly, the p85 PI3K/Lyn/CD38 association correlates with p85 phosphorylation. These results suggest that the loss of Lyn activity interrupts interactions in a proposed CD38-facilitated signaling complex that involves SLP-76, Vav1, and Lyn, and that this complex regulates the downstream phosphorylation of c-Cbl and p85 PI3K. The observed interaction between p85 PI3K, Lyn, and CD38 suggests that CD38 has direct role in assembling a Lyn kinase-containing complex that phosphorylates p85 PI3K. Together these results indicate that Lyn kinase activity regulates CD38 signaling which results in pY-c-Cbl and pY-p85 PI3K, which is associated with RA induction and PP2-enhanced differentiation. These outcomes are important in understanding how the CD38 receptor functions during differentiation, and

how it may contribute to the effects of PP2/RA co-treatment that result in differentiation enhancement.

2.3 Materials and methods

2.3-1 Cell culture

HL-60 and NB4 cells were grown in 5% serum-supplemented RPMI 1640 with 1% antibiotic/antimycotic from Invitrogen (Carlsbad, CA) and treated with 1 μM RA as previously described.[13] PP2 from EMD Chemicals (Gibbstown, NJ) was solubilized in dimethyl sulfoxide (DMSO) at 10 mM. Cells were treated with a final concentration of 10 μM with a 0.1% concentration of carrier DMSO. Dasatinib from Santa Cruz Biotechnology (Santa Cruz, CA) was solubilized in DMSO at 5 mM. Cells were treated with a final concentration of 300 nM. SFK activity inhibition was confirmed by Western blot. The concentrations of drugs were approximately 3–4 fold less than that found to cause overt toxicity in titrations monitoring cell growth with a hemocytometer and trypan blue exclusion.

2.3-2 Antibodies and reagents

Protein A/G beads used for immunoprecipitation, rabbit anti-c-Cbl, rabbit anti-Vav1, and p-Tyr antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PureProteome Protein G Magnetic Beads were from Millipore (Billerica, MA). Antibodies for GAPDH, beta-actin, p-Erk1/2, ERK1/2 (rabbit), pan-SFK416, Lyn, Fgr, Lck, Fyn, Vav1, SLP-76, pY-p55/p85 PI3K, total p85 PI3K, HRP anti-mouse, and HRP anti-rabbit were from Cell Signaling (Danvers, MA). CD38 antibody was purchased from BD Pharmingen (San Jose, CA). M-PER Mammalian Protein Extraction Reagent lysis buffer was from Pierce

(Rockford, IL). Propidium iodide, protease and phosphatase inhibitors, and DMSO were purchased from Sigma (St. Louis, MO).

2.3-3 Construction of CD38+ stable transfectants

CD38 knock-in plasmid construction and transfection were performed as previously described.[2] To maintain CD38 high expression in stable transfectants, the cells were stained with APC-conjugated anti-CD38 antibody (BD Biosciences, San Jose, CA) and sorted based on high expression of CD38 using a fluorescence activated cell sorter (FACS) flow cytometer (FACS Aria BD Biosciences). Western blotting confirmed CD38 expression.

2.3-4 Western blot analysis and immunoprecipitation

For immunoprecipitation experiments, cells were lysed as previously described.[13] Equal amounts of protein were pre-cleared with either Protein A/G beads or PureProteome Protein G Magnetic Beads. The beads were pelleted and supernatant was incubated with appropriate antibodies and fresh beads overnight. All incubations included protease and phosphatase inhibitors used for lysis with constant rotation at 4°C. Bead/antibody/protein slurries were then washed and subjected to standard SDS-PAGE analysis as previously described.[13]

2.3-5 Fluorescence resonance energy transfer (FRET)

Cells were harvested, fixed, and permeabilized as previously described.[16] Cells were resuspended in 200 µl of PBS containing 5 µl of primary rabbit anti-SLP-76 or rabbit anti-Vav1 and mouse anti-CD38 antibodies and then stained with Alexa-350 and 430-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies, respectively, from Invitrogen. The immunocomplexes were analyzed using flow cytometry (LSR II, BD

Biosciences). The FRET signal was measured as previously described.[16] The Alexa 350 emission from 325 excitation was measured reflected from a 505 longpass dichroic through a 440/40 bandpass filter. Alexa 430 emission from 488 nm excitation (from an argon ion laser) was collected through a 505 longpass dichroic and 530/30 bandpass filter. Controls with secondary antibody(s) only or secondary(s) plus donor or acceptor primary antibody were included. Cells stained with just SLP-76 or Vav1 or CD38 primary antibody and Alexa 350 or 430 respectively were used for compensation controls for spillover into all fluorescence collection channels. Timing gates on the collected fluorescence defined acceptor emission synchronized to donor excitation. FRET signals were corrected by subtraction of background fluorescence of negative controls with just secondary antibodies and compensation controls.

2.3-6 Signaling experiments

For signaling experiments with Lyn kinase inhibition cells were cultured for 48 hours with 1 μM RA, and then washed twice with serum-free RPMI 1640 media. Appropriate samples were incubated with 10 μM PP2 for one hour and all samples were incubated at 37°C with constant rotation. The indicated samples were then treated with 5 μM IB4, which was graciously provided by Fabio Malavasi, for the time points as shown.[9] For samples with RA/PP2 co-administration cells were cultured for 48 hours with 1 μM RA and 10 μM PP2. Signaling experiments were performed as described above for IB4 treatment, but the one hour PP2 pre-incubation was omitted.

2.3-7 Statistics

Three independent repeats were conducted in all experiments. Error bars represent the standard error. The student's t-test function in Microsoft Excel was used to analyze the data.

2.4 Results

2.4-1 CD38 interacts with the RA-regulated proteins Vav1 and SLP-76

We first determined if there was interaction between CD38 and SLP-76 or Vav1 and investigated whether or not ectopically expressing CD38 in the absence of RA affected these interactions. Therefore, we could compare effects that were dependent on RA treatment versus CD38 expression alone. Western blotting for total protein showed that SLP-76, Vav1, and CD38 were all upregulated by RA, with CD38 expression showing dependence on RA treatment (Figure 2.1a). CD38 overexpression in stable transfectants (CD38+) did not significantly increase Vav1 or SLP-76, indicating that upregulated expression of these proteins was dependent on RA.

Immunoprecipitation experiments show that RA-induced CD38 was able to complex with SLP-76 and Vav1, and CD38+ transfectants showed increased interaction (Figure 2.1b). RA treatment also increased interaction between Vav1 and SLP-76. Untreated CD38+ cells also showed increased Vav1/SLP-76 interaction as well, suggesting that CD38 expression regulates binding between these two proteins and facilitates a CD38/SLP-76/Vav1 signaling complex. GAPDH was used as a loading control for protein input for the SLP-76 probed membrane.

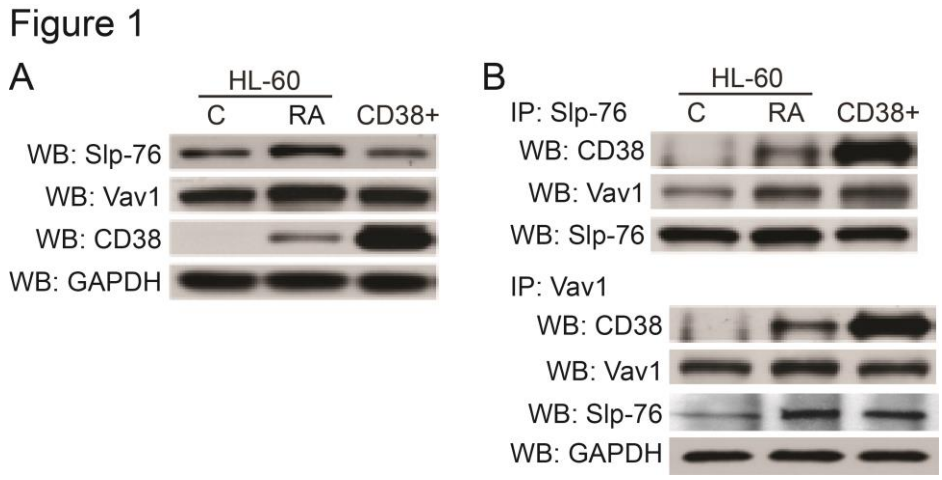


Figure 2.1: CD38 interacts with SLP-76 and Vav1. A: Western blots (WB) for protein expression of SLP-76, Vav1, and CD38 in HL-60 cells after 48 hours of RA treatment, or in untreated CD38+ transfectants. B: Immunoprecipitation (IP) of either SLP-76 or Vav1 shows interaction among CD38/SLP-76/Vav1.The IP:SLP-76/WB:SLP-76 blot shows protein loading for CD38/Vav1/SLP-76 interaction (top). The IP:Vav1/WB:Vav1 shows protein loading for CD38/Vav1 interaction, and WB for GAPDH shows total protein input for the Vav1/SLP-76 interaction (bottom IP).

2.4-2 FRET corroborates the CD38/SLP-76/Vav1 protein complex

We used FRET to confirm the interaction between CD38 and SLP-76 or Vav1. We included an additional myeloid leukemia cell line (NB4) which bears the characteristic APL t(15,17) translocation to show that these interactions are not specific to HL-60 cells.[42,43] As expected, CD38, SLP-76, and Vav1 were RA-upregulated as indicated in donor and acceptor channels (Figures 2.2a&d). FRET signals between CD38 and SLP-76 were observed in HL-60 and NB4 cells after 48 hours of RA treatment (Figures 2.2a,b,c) but not in untreated cells that did not express CD38. Likewise we detected interaction between and CD38 and Vav1 in RA-treated HL-60 and NB4 cells (Figures 2.2d,e,f).

Together, the results from co-immunoprecipitation and FRET experiments demonstrated that RA-treated HL-60 and NB4 cells showed interaction among CD38, SLP-76, and Vav1, and indicated that CD38 facilitated a SLP-76/Vav1/CD38 complex. These results prompted interest in CD38/Vav1/SLP-76 interactions with other signaling molecules that are regulated by both RA and CD38, specifically the SFK Lyn.

Figure 2

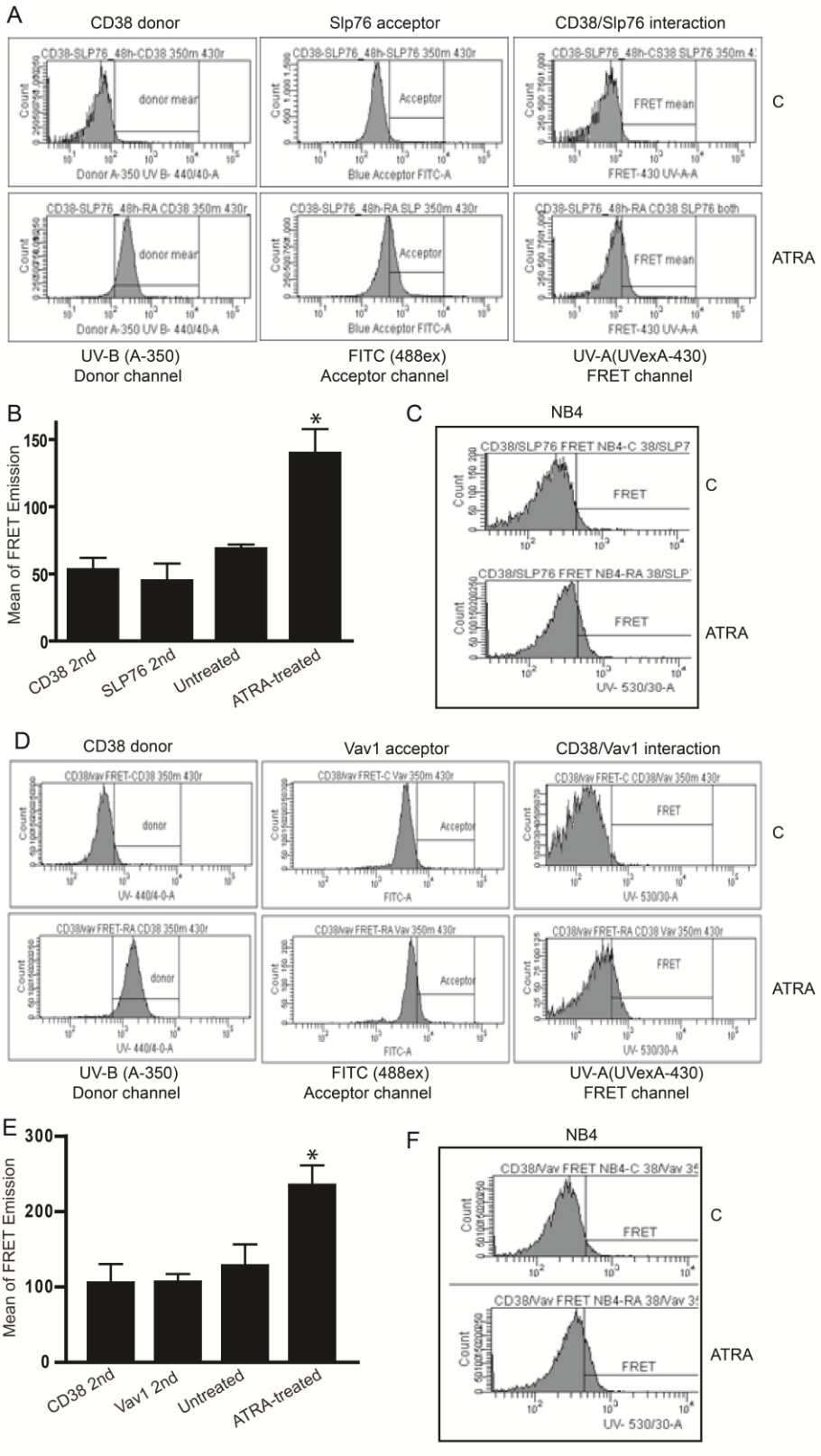

A
CD38 donor
Slp76 acceptor
CD38/Slp76 interaction
donor mean
Acceptor
FRET mean
C
ATRA
Donor A-350 UV B- 440/40-A
Blue Acceptor FITC-A
FRET-430 UV-A-A
UV-B (A-350)
Donor channel
FITC (488ex)
Acceptor channel
UV-A(UVexA-430)
FRET channel
B
Mean of FRET Emission
CD38 2nd
SLP76 2nd
Untreated
ATRA-treated
C
NB4
FRET
UV- 530/30-A
D
CD38 donor
Vav1 acceptor
CD38/Vav1 interaction
donor
Acceptor
UV- 440/4-0-A
FITC-A
UV- 530/30-A
E
F
NB4

Figure 2.2: FRET corroborates the CD38/Vav1/SLP-76 interaction. A: Flow cytometry FRET histograms confirm 48 hours of ATRA upregulates CD38 and SLP-76 expression and their interaction in HL-60 cells. B: Graph showing means of CD38/SLP-76 FRET emission in HL-60 cells. C: CD38/SLP-76 FRET interaction in NB4 cells after 48 hours of ATRA treatment. D: Flow cytometry FRET histograms confirm ATRA-upregulated CD38 and Vav1 expression and interaction in HL-60 cells. E: Graph showing means of CD38/Vav1 FRET emission in HL-60 cells. F: CD38/Vav1 FRET interaction in NB4 cells after 48 hours of ATRA treatment.

2.4-3 Lyn interacts with CD38, SLP-76, and Vav1, and kinase inhibition affects CD38-stimulated signaling

Lyn is upregulated by RA and may modulate induction therapy since siRNA against Lyn interferes with differentiation.[44] Lyn also interacts with CD38 to promote signaling.[4,34,35] Therefore, we investigated if Lyn participates in a potential CD38/Vav1/SLP-76 signaling complex. Immunoprecipitation experiments showed that Lyn was able to interact with CD38, Vav1, and SLP-76 (Figure 2.3a). RA-induced CD38 complexed with Lyn, and untreated CD38+ cells showed significantly increased interaction, indicating that CD38 alone facilitates binding. RA treatment increased interactions between Lyn/SLP-76 and Lyn/Vav1, but overexpression of CD38 in untreated cells only modestly increased Lyn/Vav1 binding. Therefore Lyn/CD38 and, to a lesser extent, Lyn/Vav1 binding is modulated by CD38 expression level but Lyn/SLP-76 binding is not.

Since CD38, SLP-76, and Vav1 also interact with c-Cbl, we probed for interaction between c-Cbl and Lyn but were unable to detect any significant evidence of association.[15,16] We also immunoprecipitated Fgr, another SFK upregulated by RA, but were only able to detect very weak binding to SLP-76 and Vav1. Western blotting for additional SFKs expressed in myeloid cells (Fyn and Lck) showed negligible protein expression (above data not shown). This is consistent with previous reports that show Lyn is the predominant SFK in myeloid leukemia cells.[36,45] Therefore, we focused our attention on the role of Lyn in CD38 signaling, which motivated interest in whether the Lyn/SFK inhibitor PP2 could modulate signaling propelled by IB4. Here, IB4 was used as a tool to stimulate CD38 signaling. As HL-60 cells express so little basal CD38, IB4 does not affect levels of the targets explored in this paper when added alone (data not shown).

We first confirmed that PP2 was effective in crippling SFK activity in HL-60 cells treated with RA for 48 hours. We incubated the indicated samples with PP2 for one hour followed by IB4 treatment as shown to evaluate if Lyn/SFK activity was enhanced by IB4, and if PP2 was able to inhibit Lyn signaling in the presence of IB4. We analyzed samples at various time points after IB4 treatment by probing Western blot membranes with an antibody that detects active site phosphorylation of all SFK members (Y416) (Figure 2.3c). We found that PP2 was able to inhibit Lyn/SFK activity after IB4 stimulation in RA-treated cells and that IB4-driven CD38 ligation did not increase Lyn/SFK kinase activity, consistent with a previous report.[28]

Lyn may cooperate with CD38 signaling molecules such as ERK, and ligation of Lyn-associated CD38 in T cells is followed by ERK and p85 PI3K phosphorylation.[33,35] We therefore evaluated whether the inhibitor affected transient IB4-induced ERK phosphorylation (Figure 2.3c). Although PP2 was able to inhibit SFK/Lyn kinase activity, it had minimal effects on ERK phosphorylation after IB4 stimulation of CD38, allowing the ERK phosphorylation to last slightly longer. We then turned our attention toward two alternative CD38 signaling targets, c-Cbl and p85 PI3K.

We immunoprecipitated c-Cbl and compared tyrosine phosphorylation status after the indicated treatments (Figure 2.3d). c-Cbl became phosphorylated after RA, and IB4 further stimulated phosphorylation. However, pre-incubation with PP2 for one hour was able to abrogate c-Cbl phoshphorylation during all time points, indicating that Lyn activity is necessary for RA- and IB4-induced c-Cbl phosphorylation. We also evaluated c-Cbl phosphorylation in untreated CD38+ cells to investigate if the effects of IB4 and PP2 were dependent on RA or CD38 expression alone (Figure 2.3d). We found that PP2 was still

able to block c-Cbl phosphorylation induced by IB4, showing the inhibitor blocked CD38-driven signaling independent of RA. It is noteworthy that the kinetics of c-Cbl phosphorylation induced by IB4 were different in CD38+ transfectants compared to RA-treated HL-60 cells, suggesting RA may temporally regulate modification of c-Cbl.

We also evaluated the effects of PP2 on p85 PI3K regulatory subunit phosphorylation. In HL-60 cells RA induced phosphorylation of the p85 PI3K subunit after 48 hours (Figure 2.3f). IB4 treatment did not result in a significant increase in phosphorylation but this may be because RA is already stimulating p-p85 PI3K, and additional phosphorylation could not be achieved. Like pY-c-Cbl, PP2 was able to block RA-induced p85 phosphorylation. Phosphorylation of the p55 isoform was not detectable. In untreated CD38+ cells, IB4 was able to stimulate the phosphorylation of the p85 PI3K subunit in the absence of RA (Figure 2.3g). We also detected an IB4-induced transient phosphorylation of the p55 PI3K isoform that was undetectable in HL-60 cells. This indicates that CD38 ectopic expression is able to regulate PI3K proteins differently than RA-induced CD38 in HL-60 cells. PP2 was able to abrogate phosphorylation of both the p55 and p85 subunit proteins, similar to its ability to block PI3K activity in HL-60 cells. Together this data suggests that Lyn kinase activity regulates CD38-stimulated phosphorylation of two downstream targets, c-Cbl and PI3K, in RA-treated HL-60 cells and CD38+ transfectants. PP2 had minimal effects on CD38-propelled ERK phosphorylation, indicating that the signaling cascade that results in p-ERK is independent of Lyn kinase activity.

Figure 3

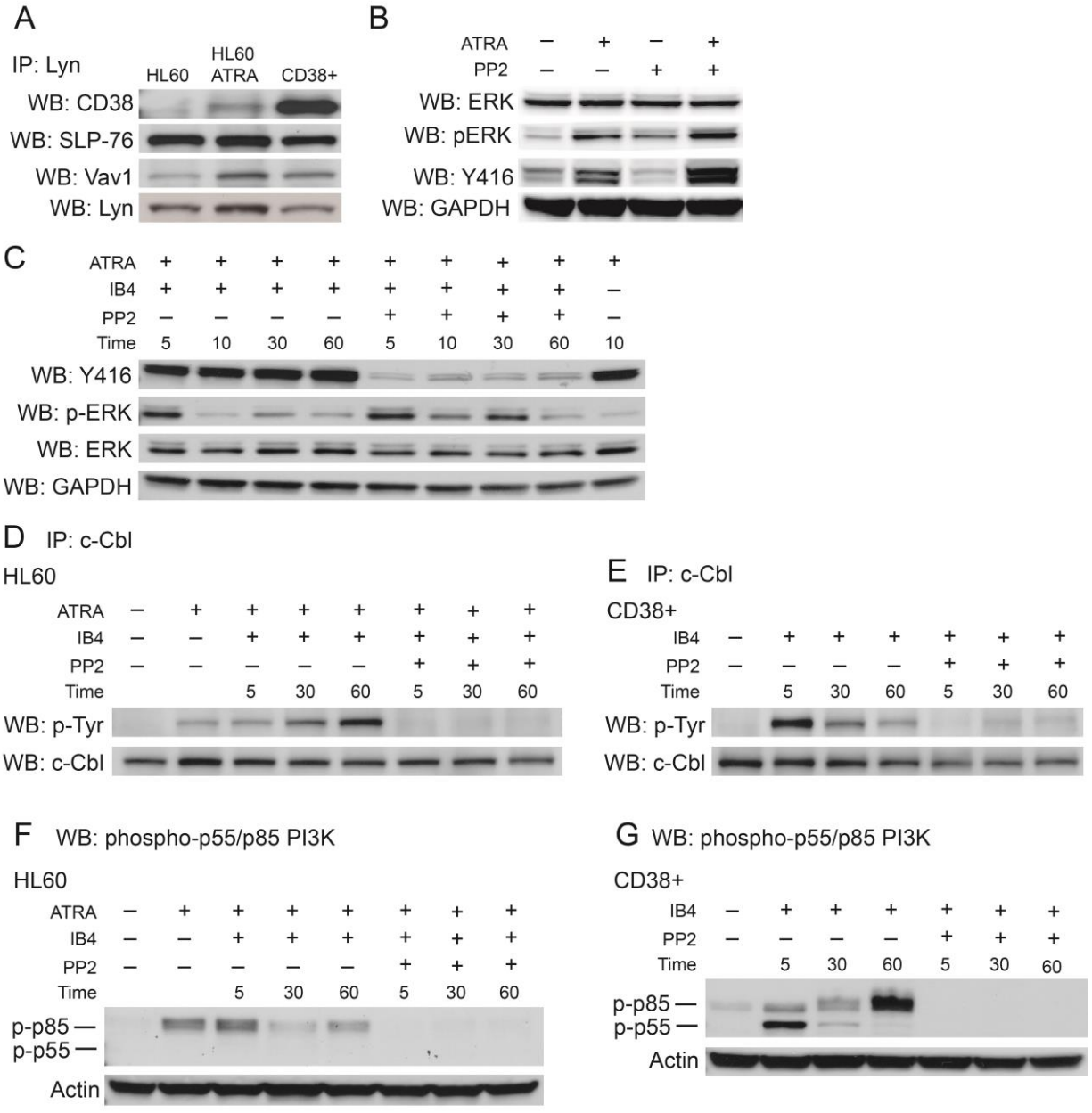

A
IP: Lyn
HL60
HL60 ATRA
CD38+
WB: CD38
WB: SLP-76
WB: Vav1
WB: Lyn
B
ATRA
PP2
WB: ERK
WB: pERK
WB: Y416
WB: GAPDH
C
ATRA
IB4
PP2
Time
5 10 30 60 5 10 30 60 10
WB: Y416
WB: p-ERK
WB: ERK
WB: GAPDH
D IP: c-Cbl
HL60
ATRA
IB4
PP2
Time
5 30 60 5 30 60
WB: p-Tyr
WB: c-Cbl
E IP: c-Cbl
CD38+
IB4
PP2
Time
5 30 60 5 30 60
WB: p-Tyr
WB: c-Cbl
F WB: phospho-p55/p85 PI3K
HL60
ATRA
IB4
PP2
Time
5 30 60 5 30 60
p-p85
p-p55
Actin
G WB: phospho-p55/p85 PI3K
CD38+
IB4
PP2
Time
5 30 60 5 30 60
p-p85
p-p55
Actin

Figure 2.3: Lyn binds CD38, SLP-76, and Vav1 and its kinase activity regulates CD38 signaling. A: Immunoprecipiation shows Lyn interaction with CD38, SLP-76, and Vav1 in HL-60 cells with and without 48 hours of ATRA treatment, and in untreated CD38+ cells. The IP:Lyn/WB:Lyn blot shows protein loading. B: Western blots for SFK/Lyn kinase basal activity in untreated, ATRA-treated, PP2-treated, and co-treated cells. All treatments lasted 48 hours. C: Western blots for SFK/Lyn kinase activity in ATRA-treated HL-60 cells using a pan-Y416 that detects active site phosphorylation in all SFK members. Cells were treated with ATRA for 48 hours, incubated with PP2 for one hour, and then stimulated by IB4 for the indicated time points. p-ERK blot shows PP2 did not significantly affect ERK phosphorylation. Total ERK and GAPDH show protein loading. D: Immunoprecipitation of c-Cbl shows tyrosine phosphorylation with indicated treatments in HL-60 cells. Cells treated with ATRA were cultured for 48 hours, cells treated with PP2 were exposed to the inhibitor for one hour, and cells were stimulated by IB4 for the indicated time points E: c-Cbl tyrosine phosphorylation in CD38+ transfectants in the absence of ATRA treated as indicated with PP2 for one hour and then stimulated with IB4. F: Western blot for phospho-p55/p85 PI3K in HL-60 cells treated as indicated. G: Western blot for phospho-p55/p85 PI3K in CD38+ transfectants treated as indicated. Actin shows protein loading in pY-p55/p85 PI3K blots.

2.4-4 Early PP2 treatment co-administered with RA protects Lyn kinase activity and permits CD38-driven c-Cbl and p85 PI3K phosphorylation

After 48 hours of RA, one hour of PP2 incubation inhibits Lyn activity (Figure 2.3). However, if PP2 treatment occurs concurrently with RA and cells are then cultured for 48 hours, Lyn kinase activity is protected.[38] Though we cannot rule out some PP2 degradation over the 48 hour treatment, based on inhibition of Y416 phosphorylation, PP2 is still effective at 48 hours.[38] The mechanism by which RA protects Lyn kinase activity from inhibition by PP2 is not known. Since one-hour PP2 incubation with RA-treated cells blocks SFK/Lyn kinase activity but PP2/RA co-treatment for 48 hours protects it, we used this strategy to evaluate if preserving Lyn activity would permit CD38 ligand-induced phosphorylation of c-Cbl and p85 PI3K.

First, we confirmed that 48 hours of co-administered PP2 and RA were able to protect Lyn activity. We compared Y416 phosphorylation in cells that were co-treated with RA and PP2 simultaneously and then cultured for 48 hours to cells that received only RA for 48 hours followed by PP2 incubation for one hour (Figure 2.4a). All RA-treated samples were treated with IB4 for the indicated time points to stimulate CD38. As expected, cells treated with RA and then later incubated with PP2 showed no Y416 phosphorylation, while cells co-treated with RA and PP2 for 48 hours permitted SFK/Lyn kinase activity.

We then evaluated if protecting Lyn activity correlated with the ability of RA and IB4 to stimulate CD38-driven c-Cbl and p85 PI3K phosphorylation. Confirming the results in Figure 3, one hour of PP2 incubation in RA-treated cells blocked p85 PI3K and c-Cbl phosphorylation in IB4-stimulated cells (Figures 2.4b&c). However, 48 hour co-treatment with RA and PP2 permitted RA- and IB4-induced c-Cbl and p85 PI3K phosphorylation.

Finally we used dasatinib, which unlike PP2 abrogates Lyn activity with RA co-administration, to evaluate if kinase inhibition blocked RA-induced c-Cbl and p85 PI3K phosphorylation (Figure 2.4d).[38] We found that dasatinib blocked c-Cbl phosphorylation and significantly decreased p85 PI3K phosphorylation, suggesting that RA-induced pY-c-Cbl requires Lyn activity and RA-upregulated p85 PI3K activity is largely dependent on Lyn.

Therefore, SFK/Lyn kinase activity regulated RA-propelled and CD38-stimulated signaling that results in phosphorylation of c-Cbl and p85 PI3K. This motivated interest in whether or not CD38 associations with other RA-induced molecules, specifically SLP-76 and Vav1, were affected by intact or lost Lyn kinase activity.

Figure 4

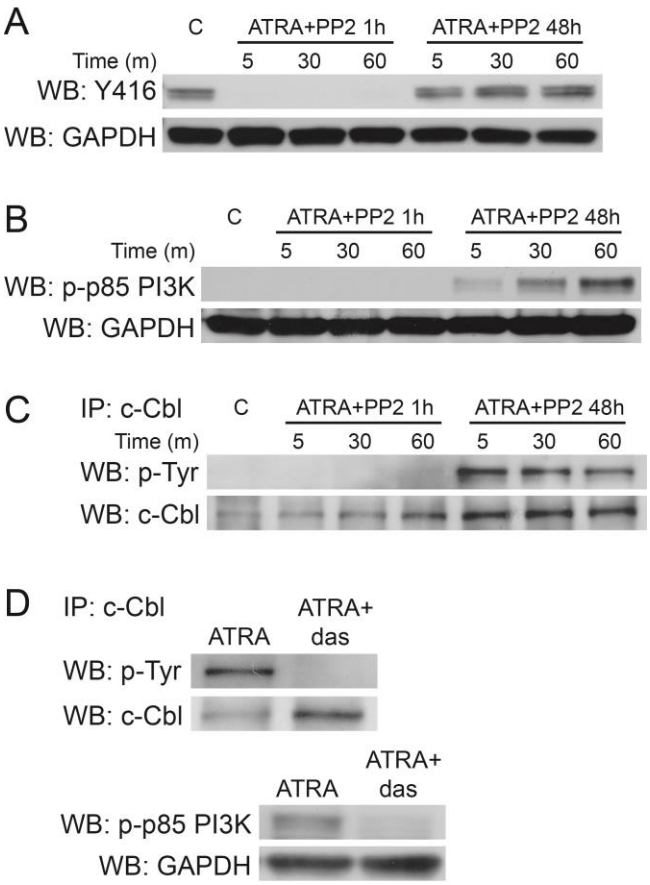

A
C
ATRA+PP2 1h
ATRA+PP2 48h
Time (m)
5
30
60
5
30
60
WB: Y416
WB: GAPDH
B
C
ATRA+PP2 1h
ATRA+PP2 48h
Time (m)
5
30
60
5
30
60
WB: p-p85 PI3K
WB: GAPDH
C
IP: c-Cbl
C
ATRA+PP2 1h
ATRA+PP2 48h
Time (m)
5
30
60
5
30
60
WB: p-Tyr
WB: c-Cbl
D
IP: c-Cbl
ATRA
ATRA+ das
WB: p-Tyr
WB: c-Cbl
ATRA
ATRA+ das
WB: p-p85 PI3K
WB: GAPDH

Figure 2.4: Protecting Lyn kinase activity preserves CD38-stimulated c-Cbl and p85 PI3K phosphorylation. A: Cells were treated with ATRA for 48 hours, incubated with PP2 for one hour or co-treated for 48 hours as indicated, and then stimulated by IB4 for the indicated time points. Western blotting shows Y416 inhibition in HL-60 cells treated with ATRA for 48 hours followed by one hour of PP2 incubation, but activation after 48 hours of ATRA/PP2 co-treatment in culture. GAPDH shows protein loading. B: Western blot for p85 PI3K phosphorylation in HL-60 cells. Samples were either untreated (C), treated with ATRA for 48 hours followed by one hour of incubation with PP2 to inhibit Lyn activity (ATRA+PP2 1h), or co-treated with ATRA and PP2 and then cultured for 48 hours (ATRA+PP2 48h) which protects Lyn kinase activity. C: c-Cbl phosphorylation in HL-60 cells after the treatments described in B. D: Cells were treated with ATRA or a combination of ATRA and dasatinib for 48 hours. c-Cbl was immunoprecipitated and membranes were probed for tyrosine phosphorylation (top). Western blotting was performed for phosphorylated p85 PI3K (bottom).

2.4-5 PP2 inhibition of c-Cbl and p85 PI3K phosphorylation coincides with decreased CD38/Lyn/SLP-76, CD38/Lyn/Vav1, and CD38/Lyn/p85 PI3K interactions

Since SLP-76 and Vav1 complex with both CD38 and Lyn, we investigated if those interactions were affected by the loss of Lyn activity. We immunoprecipitated Vav1 in samples that were either incubated with PP2 for one hour after 48 hours of RA treatment or received simultaneous PP2/RA treatment for 48 hours. All RA-treated samples were stimulated by IB4 to elicit CD38 signaling. We anticipated that an RA-induced effect seminal to protecting Lyn kinase activity from PP2 inhibition may also be relevant to interactions between CD38 and its related signaling molecules. We found that the loss of Lyn activity corresponded with a significant decrease in CD38/Vav1 and Lyn/Vav1 interaction (Figure 2.5b). This suggests that these three molecules constitute part of a signaling complex that regulates an RA-induced and CD38-stimulated modification of p85 PI3K and c-Cbl.

We also immunoprecipitated SLP-76 and probed for CD38 and Lyn. Similar to Vav1, loss of Lyn kinase activity resulted in decreased CD38/SLP-76 interaction (Figure 2.5c). Lyn/SLP-76 interaction was modestly decreased, yet the effects were not as significant as observed with Vav1 experiments. These results show that loss of c-Cbl and p85 PI3K phosphorylation, which corresponded to the inhibition of SFK/Lyn kinase activity, is associated with decreased interaction among CD38/Lyn/Vav1 and CD38/Lyn/SLP-76. This suggests that a putative signaling complex including CD38, Vav1, SLP-76, and active Lyn regulates CD38-driven signaling which characterizes RA-induced differentiation. Finally, we observed that Lyn inhibition decreased CD38/Lyn/p85 PI3K

interaction (Figure 2.5d), suggesting that CD38 facilitates a Lyn kinase-containing complex that is directly responsible for p85 PI3K phosphorylation.

Figure 5

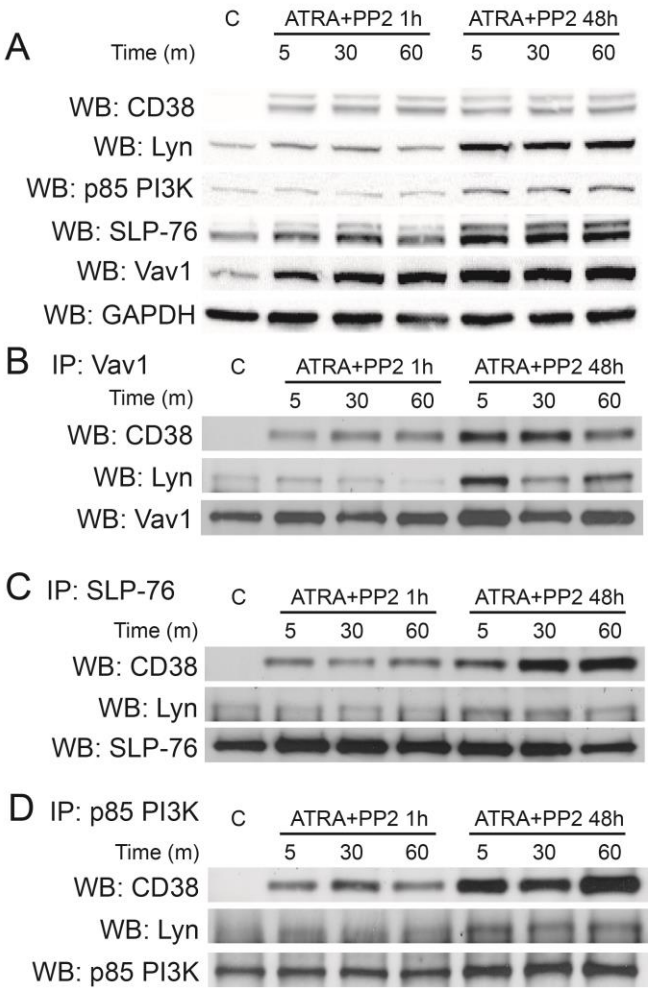

A
C
ATRA+PP2 1h
ATRA+PP2 48h
Time (m)
5 30 60 5 30 60
WB: CD38
WB: Lyn
WB: p85 PI3K
WB: SLP-76
WB: Vav1
WB: GAPDH
B IP: Vav1
C
ATRA+PP2 1h
ATRA+PP2 48h
Time (m)
5 30 60 5 30 60
WB: CD38
WB: Lyn
WB: Vav1
C IP: SLP-76
C
ATRA+PP2 1h
ATRA+PP2 48h
Time (m)
5 30 60 5 30 60
WB: CD38
WB: Lyn
WB: SLP-76
D IP: p85 PI3K
C
ATRA+PP2 1h
ATRA+PP2 48h
Time (m)
5 30 60 5 30 60
WB: CD38
WB: Lyn
WB: p85 PI3K

Figure 2.5: Lyn inhibition disrupts interactions among CD38-associated proteins. A: Cells were treated with ATRA for 48 hours, incubated with PP2 for one hour or co-treated for 48 hours as indicated, and then stimulated by IB4 for the indicated time points. Western blots show basal protein expression levels of target proteins. B: Immunoprecipitation of Vav1 shows changes in CD38 and Lyn interaction after treatments as indicated in HL-60 cells. The Vav1 blot shows protein loading. C: Immunoprecipitation of SLP-76 shows changes in CD38 and Lyn interaction after treatments as indicated. The SLP-76 blot shows protein loading. D: Immunoprecipitation of p85 PI3K shows changes in CD38 and Lyn interaction after treatments as indicated. The p85 PI3K blot shows protein loading.

2.5 Discussion

RA therapy is successful in treating t(15,17) positive APL, yet it has shown little efficacy in the treatment of other types of leukemias and cancers. The t(15,17) negative HL-60 cell line shows RA responsiveness and is used as a model to investigate how a non-APL leukemic cell can be induced to differentiate. Therefore, elucidation of signaling pathways that may confer RA responsiveness could broaden its usefulness in other diseases and aid in identifying new therapeutic molecular targets.[46]

CD38 is a leukocyte enzyme and receptor that drives MAPK signaling and differentiation when overexpressed.[2] It has important functions in RA induction, since siRNA targeting CD38 interferes with differentiation, and a CD38 mutant (CD38 Δ11–20) that is not membrane-expressed also cripples RA effectiveness.[13,14] Identifying signaling pathways orchestrated by CD38 that are involved in myeloid maturation is important in understanding how RA works.

2.5-1 CD38 may direct RA-induced differentiation via c-Cbl, Vav1, and SLP-76

In this report, we found that CD38 was able to interact with SLP-76 and Vav1, which regulate differentiation. Therefore, signaling pathways modulated by these proteins may also be propelled by the CD38 receptor. For example downregulation of Vav1 prevents RA-induced differentiation, as evidenced by loss of nucleoskeleton remodeling and maturation markers.[22–24] These reports also show that RA induction is characterized by the association of Vav1/PI3K and Vav1/SLP-76 in the nucleus and Vav1/c-Cbl in the cytosol. Those complexes could modulate signaling cascades that are important for neutrophil differentiation and may be coordinated by CD38. CD38 also associates with c-Cbl and interruption of this interaction by a c-Cbl mutation (G306E) results in loss of

MAPK signaling and RA efficacy.[15,16] These studies report that like CD38, c-Cbl binds Vav1 and SLP-76. This supports our results and suggests that membrane-expressed CD38 coordinates a putative cytosolic signaling complex involving c-Cbl, Vav1, and SLP-76, which could regulate associations with effectors including PI3K.

2.5-2 Lyn is present in a putative RA-induced signaling complex

We also report the involvement of Lyn in a proposed CD38-coordinated complex including SLP-76 and Vav1, which is significant because SFK inhibitors appear to regulate RA-induced differentiation.[38] This motivated interest in whether or not Lyn kinase activity was able to modulate signaling driven by a CD38 agonist, IB4. We evaluated three targets of CD38: ERK, c-Cbl, and the p85/p55 PI3K regulatory subunit. Cells were treated with RA for 48 hours to induce CD38, and then treated with the SFK inhibitor PP2 for one hour followed by IB4. While PP2 is not specific to Lyn, many of the other SFKs that are affected at the concentration used do not appear to play important roles in RA-induced differentiation of HL-60 cells; Fyn and Lck levels were negligible and activated Fgr was not detected regardless of treatment with RA, PP2, dasatinib, or cotreatment with RA plus either inhibitor.[38] We also used CD38+ transfectants in these experiments to evaluate if there were signaling effects that were specific to RA or CD38 expression alone.

2.5-3 Lyn kinase activity is necessary for RA-induced c-Cbl and p55/p85 PI3K, but not ERK, phosphorylation

Since CD38 and c-Cbl interact and both drive MAPK signaling when overexpressed, it is possible that they cooperatively contribute to the persistent ERK phosphorylation that is characteristic of RA treatment.[15,16,31,47] However it appears that CD38-propelled ERK phosphorylation is not mediated by Lyn kinase activity since PP2 failed to affect MAPK

signaling in RA-treated HL-60 cells. In addition, we failed to detect interaction between Lyn and c-Cbl, suggesting that CD38 and c-Cbl may cooperate to propel MAPK signaling independent of Lyn, or that Lyn/c-Cbl interaction is labile. Alternatively, it is possible that Lyn participates in CD38-driven MAPK signaling by serving as a scaffold or by facilitating signaling complex assembly. For example, PP2/RA co-treatment for 48 hours enhances Lyn expression along with Lyn/c-Raf and c-Raf/ERK interaction, and c-Raf C-terminal domain phosphorylation. These events may be facilitated by Lyn.[38,48,49] Therefore the role of Lyn in CD38-driven MAPK signaling and orchestration may involve the potential for Lyn to act as a scaffold, which appears to be separate from kinase activity.

In contrast, Lyn inhibition completely abrogated RA- and CD38-driven c-Cbl and p55/p85 PI3K phosphorylation, showing that Lyn regulates these events in both RA-treated HL-60 cells and CD38+ transfectants. It is interesting that co-administration of RA and PP2 followed by 48 hours of culture protects Lyn from the effects of PP2. Protecting Lyn kinase activity permitted CD38 ligand-induced pY-c-Cbl and pY-p85 PI3K in HL-60 cells. Dasatinib, which unlike PP2 inhibits Lyn when co-administered with RA, blocked c-Cbl phosphorylation and impeded pY-p85 PI3K. Therefore, Lyn kinase activity regulates CD38-stimulated signaling and RA-induced phosphorylation events that may be driven by CD38.

2.5-4 Lyn kinase activity is necessary for the formation of several RA-induced CD38-associated complexes

We also show that the loss of Lyn kinase activity coincided with a loss in interaction among CD38/Lyn/SLP-76, CD38/Lyn/Vav1, and CD38/Lyn/p85 PI3K. This suggests that the assembly of these CD38-associated complexes, which are likely involved in CD38

effector signaling after RA treatment, is partially dependent on Lyn kinase activity. It also indicates that CD38 participates in the assembly of a Lyn kinase-containing complex that may result in the direct phosphorylation of p85 PI3K.

It is interesting that co-treatment with PP2 and RA, which significantly enhances differentiation, preserves Lyn kinase activity.[38] The mechanism by which RA protects Lyn from PP2 inhibition is not known. In contrast dasatinib is able to inhibit Lyn in the presence of RA and also abrogates c-Cbl tyrosine phosphorylation and significantly decreases p85 PI3K subunit activity driven by RA. Dasatinib still enhances differentiation with RA co-treatment, but to a significantly lesser extent than PP2. This suggests signaling that characterizes normal RA induction in HL-60 cells that is mediated by Lyn kinase activity may help drive differentiation and confer the increased effectiveness of PP2 compared to dasatinib.

In sum, we report a CD38/SLP-76/Vav1 interaction and found that Lyn also interacts with these proteins. We found that CD38-propelled p85 PI3K and c-Cbl phosphorylation, which is characteristic of RA-induced differentiation, is mediated by Lyn kinase activity. To our knowledge, this is the first report that Lyn regulates RA- and CD38-transduced signaling in myeloid leukemia cells and elucidates how CD38 partner molecules including SLP-76 and Vav1 may regulate differentiation during RA or RA/PP2 co-treatment.

REFERENCES

1. Drach, J. *et al.* Retinoic acid-induced expression of CD38 antigen in myeloid cells is mediated through retinoic acid receptor-alpha. *Cancer Res.* **54,** 1746–1752 (1994).
2. Lamkin, T. J. *et al.* Retinoic acid-induced CD38 expression in HL-60 myeloblastic leukemia cells regulates cell differentiation or viability depending on expression levels. *J. Cell. Biochem.* **97,** 1328–1338 (2006).
3. Kontani, K. *et al.* Tyrosine phosphorylation of the c-cbl proto-oncogene product mediated by cell surface antigen CD38 in HL-60 cells. *J. Biol. Chem.* **271,** 1534–1537 (1996).
4. Kitanaka, A., Ito, C., Coustan-Smith, E. & Campana, D. CD38 ligation in human B cell progenitors triggers tyrosine phosphorylation of CD19 and association of CD19 with lyn and phosphatidylinositol 3-kinase. *J. Immunol.* **159,** 184–192 (1997).
5. Kitanaka, A. *et al.* CD38-mediated signaling events in murine pro-B cells expressing human CD38 with or without its cytoplasmic domain. *J. Immunol.* **162,** 1952–1958 (1999).
6. Zubiaur, M., Izquierdo, M., Terhorst, C., Malavasi, F. & Sancho, J. CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-ζ/ζ-associated protein-70 signaling pathways in Jurkat T lymphocytes. *J. Immunol.* **159,** 193–205 (1997).
7. Zubiaur, M., Guirado, M., Terhorst, C., Malavasi, F. & Sancho, J. The CD3-gamma delta epsilon transducing module mediates CD38-induced protein-tyrosine kinase and mitogen-activated protein kinase activation in Jurkat T cells. *J. Biol. Chem.* **274,** 20633–20642 (1999).
8. Zubiaur, M. *et al.* CD38 is associated with lipid rafts and upon receptor stimulation leads to Akt/protein kinase B and Erk activation in the absence of the CD3-ζ immune receptor tyrosine-based activation motifs. *J. Biol. Chem.* **277,** 13–22 (2002).
9. Funaro, A. *et al.* Involvement of the multilineage CD38 molecule in a unique pathway of cell activation and proliferation. *J. Immunol.* **145,** 2390–2396 (1990).
10. Lund, F. E. *et al.* CD38 induces apoptosis of a murine pro-B leukemic cell line by a tyrosine kinase-dependent but ADP-ribosyl cyclase- and NAD glycohydrolase-independent mechanism. *Int. Immunol.* **18,** 1029–1042 (2006).
11. Malavasi, F. *et al.* Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol. Rev.* **88,** 841–886 (2008).
12. Kumagai, M. *et al.* Ligation of CD38 suppresses human B lymphopoiesis. *J. Exp. Med.* **181,** 1101–1110 (1995).
13. Congleton, J., Jiang, H., Malavasi, F., Lin, H. & Yen, A. ATRA-induced HL-60 myeloid leukemia cell differentiation depends on the CD38 cytosolic tail needed for membrane localization, but CD38 enzymatic activity is unnecessary. *Exp. Cell Res.* **317,** 910–919 (2011).
14. Munshi, C. B., Graeff, R. & Lee, H. C. Evidence for a causal role of CD38 expression in granulocytic differentiation of human HL-60 cells. *J. Biol. Chem.* **277,** 49453–49458 (2002).
15. Shen, M. & Yen, A. c-Cbl interacts with CD38 and promotes retinoic acid-induced

differentiation and G0 arrest of human myeloblastic leukemia cells. *Cancer Res.* **68,** 8761–8769 (2008).
16. Shen, M. & Yen, A. c-Cbl tyrosine kinase-binding domain mutant G306E abolishes the interaction of c-Cbl with CD38 and fails to promote retinoic acid-induced cell differentiation and G0 arrest. *J. Biol. Chem.* **284,** 25664–25677 (2009).
17. Adapala, N. S. *et al.* The loss of Cbl-phosphatidylinositol 3-kinase interaction perturbs RANKL-mediated signaling, inhibiting bone resorption and promoting osteoclast survival. *J. Biol. Chem.* **285,** 36745–36758 (2010).
18. Adapala, N. S., Barbe, M. F., Langdon, W. Y., Tsygankov, A. Y. & Sanjay, A. Cbl-phosphatidylinositol 3 kinase interaction differentially regulates macrophage colony-stimulating factor-mediated osteoclast survival and cytoskeletal reorganization. *Ann. N.Y. Acad. Sci.* **1192,** 376–384 (2010).
19. Ohashi, E., Kogai, T., Kageehika, H. & Brent, G. A. Activation of the PI3 kinase pathway by retinoic acid mediates sodium/iodide symporter induction and Iodide transport in MCF-7 breast cancer cells. *Cancer Res.* **69,** 3443–3450 (2009).
20. Scholl, S. *et al.* Additive effects of PI3-kinase and MAPK activities on NB4 cell granulocyte differentiation: Potential role of phosphatidylinositol 3-kinase γ. *J. Cancer Res. Clin. Oncol.* **134,** 861–872 (2008).
21. Yen, A., Varvayanis, S., Smith, J. L. & Lamkin, T. J. Retinoic acid induces expression of SLP-76: Expression with c-FMS enhances ERK activation and retinoic acid-induced differentiation/G0 arrest of HL-60 cells. *Eur. J. Cell Biol.* **85,** 117–132 (2006).
22. Bertagnolo, V. *et al.* Vav promotes differentiation of human tumoral myeloid precursors. *Exp. Cell Res.* **306,** 56–63 (2005).
23. Bertagnolo, V. *et al.* Vav1 modulates protein expression during ATRA-lnduced maturation of APL-derived promyelocytes: A proteomic-based analysis. *J. Proteome Res.* **7,** 3729–3736 (2008).
24. Bertagnolo, V. *et al.* Requirement of tyrosine-phosphorylated Vav for morphological differentiation of all-trans-retinoic acid-treated HL-60 cells. *Cell Growth Differ* **12,** 193–200 (2001).
25. Bertagnolo, V. *et al.* Association of PI 3-K with tyrosine phosphorylated Vav is essential for its activity in neutrophil-like maturation of myeloid cells. *Cell. Signal.* **16,** 423–433 (2004).
26. Kitanaka, A., Ito, C., Nishigaki, H. & Campana, D. CD38-mediated growth suppression of B-cell progenitors requires activation of phosphatidylinositol 3-kinase and involves its association with the protein product of the c-cbl proto-oncogene. *Blood* **88,** 590–598 (1996).
27. Matsuo, T. *et al.* Association of phosphatidylinositol 3-kinase with the proto-oncogene product Cbl upon CD38 ligation by a specific monoclonal antibody in THP-1 cells. *FEBS Lett.* **397,** 113–116 (1996).
28. Silvennoinen, O. *et al.* CD38 signal transduction in human B cell precursors. Rapid induction of tyrosine phosphorylation, activation of syk tyrosine kinase, and phosphorylation of phospholipase C-gamma and phosphatidylinositol 3-kinase. *J. Immunol.* **156,** 100–107 (1996).
29. Lewandowski, D. *et al.* Phosphatidylinositol 3-kinases are involved in the all-trans retinoic acid-induced upregulation of CD38 antigen on human haematopoietic cells.

Br. J. Haematol. **118,** 535–544 (2002).
30. Traverse, S., Gomez, N., Paterson, H., Marshall, C. & Cohen, P. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem. J.* **288** (Pt 2) 351–355 (1992).
31. Yen, A, Roberson, M. S., Varvayanis, S. & Lee, A. T. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res.* **58,** 3163–3172 (1998).
32. Wang, J. & Yen, A. A MAPK-positive feedback mechanism for BLR1 signaling propels retinoic acid-triggered differentiation and cell cycle arrest. *J. Biol. Chem.* **283,** 4375–4386 (2008).
33. Rodríguez-Alba, J. C., Moreno-García, M. E., Sandoval-Montes, C., Rosales-Garcia, V. H. & Santos-Argumedo, L. CD38 induces differentiation of immature transitional 2 B lymphocytes in the spleen. *Blood* **111,** 3644–3652 (2008).
34. Yasue, T. *et al.* A critical role of Lyn and Fyn for B cell responses to CD38 ligation and interleukin 5. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 10307–10312 (1997).
35. Zumaquero, E. *et al.* Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signaling complexes containing CD81, Hsc-70 and Lyn. *Exp. Cell Res.* **316,** 2692–2706 (2010).
36. Kropf, P. L., Wang, L., Zang, Y., Redner, R. L. & Johnson, D. E. Dasatinib promotes ATRA-induced differentiation of AML cells. *Leukemia* **24,** 663–665 (2010).
37. Miranda, M. B., Redner, R. L. & Johnson, D. E. Inhibition of Src family kinases enhances retinoic acid induced gene expression and myeloid differentiation. *Mol. Cancer Ther.* **6,** 3081–3090 (2007).
38. Congleton, J., MacDonald, R. & Yen, A. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. *Leukemia* **26,** 1180–1188 (2012).
39. Hanke, J. H. *et al.* Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor: Study of Lck- and FynT-dependent T cell activation. *J. Biol. Chem.* **271,** 695–701 (1996).
40. Ben-Sasson, H. *et al.* All-trans-retinoic acid mediates changes in PI3K and retinoic acid signaling proteins of leiomyomas. *Fertil. Steril.* **95,** 2080–2086 (2011).
41. Lande, R. *et al.* CD38 ligation plays a direct role in the induction of IL-1β, IL-6, and IL-10 secretion in resting human monocytes. *Cell. Immunol.* **220,** 30–38 (2002).
42. Fagioli, M. *et al.* Alternative splicing of PML transcripts predicts coexpression of several carboxy-terminally different protein isoforms. *Oncogene* **7,** 1083–1091 (1992).
43. Biondi, A. *et al.* RAR-alpha gene rearrangements as a genetic marker for diagnosis and monitoring in acute promyelocytic leukemia. *Blood* **77,** 1418–1422 (1991).
44. Katagiri, K. *et al.* Lyn and Fgr protein-tyrosine kinases prevent apoptosis during retinoic acid-induced granulocytic differentiation of HL-60 cells. *J. Biol. Chem.* **271,** 11557–11562 (1996).
45. Dos Santos, C. *et al.* A critical role for Lyn in acute myeloid leukemia. *Blood* **111,** 2269–2279 (2008).

46. Malavasi, F. Editorial: CD38 and retinoids: a step toward a cure. *J. Leukoc. Biol.* **90,** 217–219 (2011).
47. Lamkin, T. J., Chin, V. & Yen, A. All-trans retinoic acid induces p62DOK1 and p56DOK2 expression which enhances induced differentiation and G0 arrest of HL-60 leukemia cells. *Am. J. Hematol.* **81,** 603–615 (2006).
48. Brummer, T., Naegele, H., Reth, M. & Misawa, Y. Identification of novel ERK-mediated feedback phosphorylation sites at the C-terminus of B-Raf. *Oncogene* **22,** 8823–8834 (2003).
49. Dougherty, M. K. *et al.* Regulation of Raf-1 by direct feedback phosphorylation. *Mol. Cell* **17,** 215–224 (2005).

Chapter 3 Foreword

The work in this chapter represents a significant undertaking by both Dr. Jonathan H. Shrimp and Robert MacDonald first to test for effects of induced homodimerization of CD38 on RA-induced differentiation and second to test effects of loss of the protein on the process. Dr. Shrimp continued work started by Dr. Hong Jiang on creating the linker, first trying to use a shortened form based on F-araNMN rather than F-araNAD$^+$, but after failure to induce a high degree of CD38 dimerization had to revert to synthesizing the F-araNAD$^+$-based compound, shown in Figure 3.1. He demonstrated that this compound was able to induce a high degree of dimerization of purified CD38, shown in Figure 3.2A. Following his graduation, batches of the linker were synthesized by Dr. Jiang and Lu Zhang. Downstream work using the linker (Figures 3.2B-D and 3.3), as well as the CRISPR/Cas9 work, (Figures 3.4-3.6) was performed by Robert MacDonald. Aside from sections describing the synthesis of the linker, which was written by Dr. Shrimp, the manuscript was written by Robert MacDonald and edited by all authors. The paper was ultimately published and the citation is below:

MacDonald, R. J., Shrimp, J. H., Jiang, H., Zhang, L., Lin, H. & and Yen, A. Probing the requirement for CD38 in retinoic acid-induced HL-60 cell differentiation with a small molecule dimerizer and genetic knockout. *Sci. Rep.* **12,** 17406 (2017).

Chapter 3: Probing the requirement for CD38 in retinoic acid-induced HL-60 cell differentiation with a small molecule dimerizer and genetic knockout

3.1 Abstract

CD38 is an ectoenzyme and receptor with key physiological roles. It metabolizes NAD^+ to adenosine diphosphate ribose (ADPR) and cyclic ADPR, regulating several processes including calcium signalling. CD38 is both a positive and negative prognostic indicator in leukaemia. In all-*trans* retinoic acid (RA)-induced differentiation of acute promyelocytic leukaemia and HL-60 cells, CD38 is one of the earliest and most prominently upregulated proteins known. CD38 overexpression enhances differentiation, while morpholino- and siRNA-induced knockdown diminishes it. CD38, via Src family kinases and adapters, interacts with a MAPK signalling axis that propels differentiation. Motivated by evidence suggesting the importance of CD38, we sought to determine whether it functions via dimerization. We created a linker based on the suicide substrate arabinosyl-2'-fluoro-2'-deoxy NAD^+ (F-araNAD$^+$), dimeric F-araNAD$^+$, to induce homodimerization. CD38 homodimerization did not affect RA-induced differentiation. Probing the importance of CD38 further, we created HL-60 cell lines with CRISPR/Cas9-mediated CD38 truncations. Deletion of its enzymatic domain did not affect differentiation. Apart from increased RA-induced CD11b expression, ablation of all but the first six amino acids of CD38 affected neither RA-induced differentiation nor associated signalling. Although we cannot discount the importance of this peptide, our study indicates that CD38 is not necessary for RA-induced differentiation.

3.2 Introduction

CD38 is a type II transmembrane ectoenzyme and receptor with roles in numerous physiological processes and malignancies. Through its extracellular enzymatic activity,

which converts NAD^+ to adenosine diphosphate-ribose (ADPR) and, to a small extent, cyclic ADPR, CD38 regulates calcium signalling and thus affects many processes such as bone metabolism, insulin secretion, and oxytocin secretion.[1–5] CD38 also acts as a receptor and is involved in B and T lymphocyte activation, as well as leukocyte adhesion.[6–8] It is a negative prognostic indicator in HIV and chronic lymphocytic leukaemia, but a positive prognostic indicator in acute myeloid leukaemia.[9–12] Its attributes as a receptor giving rise to these different effects are ergo of interest to both pathogenesis and therapy.

Several pieces of evidence suggest that CD38 may be a key driver of all-*trans* retinoic acid (RA)-induced differentiation. In HL-60 acute myeloid leukaemia cells, a model for RA-induced differentiation, RA induces dramatic upregulation of CD38 mRNA and protein expression within 8 h.[13–16] Overexpression of CD38 enhances RA-induced myelo-monocytic differentiation evidenced by markers including CD11b expression, G_1/G_0 cell cycle arrest, and inducible reactive oxygen species production.[17] Conversely, morpholino- and siRNA-mediated knockdown of CD38 cripples RA-induced differentiation.[18] CD38 interacts with a number of receptor-regulated/associated signalling molecules including c-Cbl, Slp76, Vav1, and Lyn, several adapters and a Src family kinase.[19–21] These proteins also interact with one another.[19–21] Further, Lyn is linked to c-Raf and ERK, members of a MAPK signalling cascade necessary for RA-induced differentiation.[22–24] Ligation of CD38 using its ligand, CD31, or anti-CD38 monoclonal antibodies induces activation of a multitude of signalling proteins, including c-Cbl, p85 PI3K, c-Raf, and ERK, as well as secretion of cytokines such as IL-6 and IL-10.[8,20,25–29]

These clues drove us to investigate what CD38 receptor functions might contribute to RA-induced differentiation. We considered three classical receptor signal transduction mechanisms by which CD38 might act. One is via signalling dependent on its intracellular domain. The cytosolic tail, however, is very short, comprising only 21 amino acids. Another is through its extracellular domain, which possesses its catalytic activity. A previous report discounted this, demonstrating that CD38 catalytic activity is not necessary for RA-induced differentiation.[30] Finally, CD38 might propel RA-induced differentiation through dimerization-induced signalling, which might depend on extracellular and intracellular domains and be induced by its ligand, CD31, or by CD38 antibodies. We viewed dimerization as particularly attractive since it is not only one of the most common mechanisms by which transmembrane receptors function, but it could also explain the ensemble of interactions between CD38 and differentiation-driving cytosolic signalling proteins. Since CD38 has a short cytosolic tail, dimerization would allow for a larger area upon which these interactions could occur. A prior study has also found that, in murine B lymphocytes, CD38 is expressed as non-covalently associated homodimers.[31] We thus opted to investigate dimerization as a potential differentiation-driving signalling modality of CD38.

To assess the effects of CD38 dimerization on RA-induced differentiation, we used a small molecule dimerizer of CD38 to induce CD38 homodimer formation. We found that CD38 dimerization did not affect RA-induced differentiation in HL-60 cells. To further probe the role of CD38 in the process, we then used the CRISPR/Cas9 system to disrupt the CD38 gene in HL-60 cells. Surprisingly, we found that apart from an increase in CD11b in one of three lines, ablation of all but six amino acids of the wild-type protein had

no effect on RA-induced differentiation, suggesting that the CD38 is not necessary for the process to occur.

3.3 Materials and methods

3.3-1 Cell culture

Reagents, unless specified otherwise, were purchased from commercial suppliers in the highest purity available and used as supplied. HL-60 human myeloblastic leukaemia cells and stable transfectant cell lines (CRISPR 1, CRISPR 2, CRISPR 3) were cultured in RPMI 1640 supplemented with 5% heat-inactivated foetal bovine serum (GE Healthcare, Chicago, IL) and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA) in a 5% CO_2 humidified atmosphere at 37^{o}C. RA (Sigma, St. Louis, MO) was solubilized in absolute ethanol. A final concentration of 1 µM was used. Arabinosyl-2'-fluoro-2'-deoxy NAD^+ (F-araNAD$^+$) and dimeric F-araNAD$^+$ (dF-araNAD$^+$) were used at final concentrations of 1 µM.

3.3-2 Antibodies and reagents

PE-conjugated CD38 (clone HIT2, catalogue number 555460, lot 5027616), APC-conjugated CD11b (clone ICRF44, catalogue number 550019, lot 5051946), CD38 for western blotting (clone 22/CD38, catalogue number 611114, lot 3295551), and CD38 antibodies for direct conjugation to Alexa Fluors (clone HIT2, catalogue number 555458, lot 4080720) were purchased from Becton Dickinson (Franklin Lakes, NJ). GAPDH (clone D16H11, catalogue number 5174S, lot 6), Lyn (catalogue number 2732S, lot 4), Slp76 (catalogue number 4958S, lot 2), Vav1 (catalogue number 2502S, lot 2), total MEK 1/2 (catalogue number 9122L, lot 14), phospho-MEK 1/2 (clone 41G9, catalogue number 9154S, lot 14), total ERK 1/2 (clone 137F5, catalogue number 4695S, lot 14), p47^{phox}

(clone D21F6, catalogue number 4301S, lot 1), HRP-linked anti-mouse (catalogue number 7076S, lot 32), and HRP-linked anti-rabbit (catalogue number 7074S, lot 26) antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Anti-c-Cbl (clone C-15, catalogue number sc-170, lot H0414) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 and 594 succinimidyl esters, Stbl3 competent cells, and slide-a-lyzer MINI 20 kDa molecular weight cutoff (MWCO) dialysis units were purchased from Thermo Scientific (Waltham, MA). The eCRISP small guide RNA (sgRNA) tool was used to select sgRNA specific to CD38 and oligos were purchased from IDT (Coralville, IA).[32]

3.3-3 Dimerization of purified CD38

CD38 was expressed and purified as previously described.[33,34] 5 µM purified CD38 was incubated for 30 min at 37°C with or without 20 µM dF-araNAD$^+$ in 10 µL reaction buffer (25 mM HEPES, 50 mM NaCl, pH 7.4) and then mixed with 2 µL 6x protein loading buffer. Samples were heated at 100°C for 7 min, resolved by SDS-PAGE, and stained with Coomassie blue.

3.3-4 Direct conjugation of Alexa Fluors to primary antibodies

Conjugation of Alexa Fluor succinimidyl esters to CD38 primary antibodies was performed using the manufacturer's protocol. Briefly, 30 µL 0.75 M sodium bicarbonate, pH 8.3, was added to 200 µL CD38 antibody. 1 mg of Alexa Fluor was dissolved in 100 µL DMSO, and 20 µL Alexa Fluor 488 or 594 was added to one vial of primary antibody while vortexing. The tubes were then shaken at room temperature for 1 h at 200 RPM. 20 µL 1.5 M hydroxylamine, pH 8.5, was then added to each tube and they were shaken for an

additional 1 h. Finally, the conjugated antibodies were dialyzed in 2 L of PBS in the dark at room temperature for 2 h.

3.3-5 Fluorescence resonance energy transfer

HL-60 cells were cultured for 24 h with 1 µM RA as indicated. At 23 h, F-araNAD$^+$ or dF-araNAD$^+$ was added to appropriate flasks. At 24 h, 1x10^6 cells from each sample were centrifuged at 700 RPM for 5 min. Samples were washed with PBS twice before being resuspended in 200 µL PBS containing 5 µL of a 1:1 mixture of Alexa Fluor 488- and 594-conjugated CD38 antibodies. Samples were incubated for 1 h at 37^{o}C in the dark. Samples were analysed using a Becton Dickinson FACS Aria III SORP (San Jose, CA). To measure the FRET signal, a 488 nm laser line was used to excite Alexa Fluor 488, which in turn excited Alexa Fluor 594; emission from Alexa Fluor 594 was collected through a 600 nm longpass dichroic mirror and a 610/20 nm bandpass filter. Unstained controls and controls stained with Alexa Fluor 488-conjugated CD38 or Alexa Fluor 594-conjugated CD38 alone were used to set compensation values.

3.3-6 Western blot analysis

Cells were pelleted, washed twice with PBS, and lysed with ice cold M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) with protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Samples were incubated overnight at -80^{o}C and debris was pelleted. Protein concentration was determined using the Pierce BCA Protein Assay according to the manufacturer's protocol. Lysate was subjected to standard SDS-PAGE, using 25 µg of lysate per lane under denaturing conditions. Membranes were blocked with 5% dry nonfat milk in PBS and probed with antibodies described above. Images were

captured on a Bio-Rad VersaDoc MP 5000 and analysed with Quantity One software (Hercules, CA).

3.3-7 Flow cytometric phenotypic analysis

Immunostaining for CD11b and CD38 was performed as previously described and analysed using a Becton Dickinson LSR II flow cytometer (San Jose, CA) [35]. Gating was set to exclude 95% of the untreated wild-type HL-60 samples. Propidium iodide (PI) cell cycle analysis was performed as previously described.[35,36]

3.3-8 Generation of stable transfectants

CD38 was targeted for CRISPR/Cas9-mediated disruption using sgRNA sequences generated by the E-CRISP tool.[32] Sequences were designed for use in the pLentiCRISPR v2 plasmid (Addgene #52961) (Addgene, Cambridge, MA) and obtained from IDT (Coralville, IA). Three sgRNA sequences were used (CRISPR 1, CRISPR 2, and CRISPR 3) to minimize off-target effects. The sequences are: CRISPR 1 (F: 5'-CACCGCGCCAGCAGTGGAGCGGTCC-3'; R: 5'-AAACGGACCGCTCCACTGCTGGCGC-3'), CRISPR 2 (F: 5'-CACCGCAGGGTTTGTCCCCGGACAC-3'; R: 5'-AAACGTGTCCGGGGACAAACCCTGC-3'), and CRISPR 3 (F: 5'-CACCGCTCCACTGCTGGCGCCACCT-3'; R: 5'-AAACAGGTGGCGCCAGCAGTGGAGC-3'). These sequences were each cloned into pLentiCRISPR v2 following the depositor's protocol.

Lentiviral particles were produced using 2.5 µg pMD2.g (Addgene #12259), 7.5 µg psPAX2 (Addgene #12260), and 10 µg pLentiCRISPR v2 with CRISPR 1, 2, or 3 inserted. These were co-transfected into HEK 293T cells at roughly 50% confluence in 10 cm cell

culture dishes with DMEM and 10% FBS using TransIT-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol in a 3:2 ratio of reagent (µL) to plasmid (µg). After 48 h, media containing viral particles was collected and 5 mL of additional media was added to the dishes for 24 h until final collection. The 15 mL total viral media was concentrated using Amicon Ultra (Millipore, Billerica, MA) centrifugal filters with 30,000 kDa MWCO. Concentrated viral media was stored at -80^{o}C until use.

Transduction of HL-60 cells with the lentiviral particles was performed in 6-well plates. 100 µL concentrated viral particles was added to 5x10^{4} cells in 1 mL RPMI 1640 with 5% heat-inactivated FBS. After 72 h, transduced cells were put into 10 mL flasks and cultured in RPMI 1640 with 5% FBS and selected for 3 weeks in 400 ng/mL puromycin. The cell lines were evaluated for CD38 expression following 24 h treatment with 1 µM RA. In all cases, while the majority did not upregulate CD38 in response to RA treatment, there was a population of 15-30% that expressed CD38 levels comparable to wild-type HL-60 cells. Thus, a single cell sort into a 96-well plate was performed on each cell line using a Becton Dickinson FACS Aria III SORP. Upon confluence, each well was transferred to a 24-well plate and RA-induced CD38 expression was assessed as before. Several wells per cell line which did not express CD38 were pooled and designated CRISPR 1, CRISPR 2, and CRISPR 3 according to the sgRNA used.

3.3-9 mRNA isolation and sequencing

Total RNA was extracted from CRISPR 1, CRISPR 2, and CRISPR 3 cell lines using a Qiagen RNeasy Plus Mini kit following the manufacturer's protocol (Hilden, Germany). RT-PCR was performed using Invitrogen SuperScript III One Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA) on a PTC-100 thermocycler from MJ Research (Waltham,

MA). Forward (5'-AGAAGGGGAGGTGCAGTTTC-3') and reverse (5'-TGTTGCAAGGTACGGTCTGA-3') primers for CD38 were purchased from IDT (Coralville, IA). Products were separated on a 1.5% acrylamide gel and appropriate bands were excised and purified with a Qiagen QIAquick Gel Extraction Kit according to the manufacturer's protocol. Because the lines were established from several populations, there were two bands corresponding to slightly different CD38 mutants in the CRISPR 2 line, denoted upper and lower. DNA samples were sequenced at Cornell University's Life Sciences Core Laboratory. DNA sequences were translated to amino acid sequences using ExPASY Translate and compared to the sequence of wild-type CD38.

3.3-10 Detection of CD38 enzymatic activity

ADP-ribosyl cyclase activity was detected by fluorometric analysis of the NGD^+ metabolic product cyclic GDP ribose (cGDPR) using a spectrofluorometer (Horiba Jobin Yvon FluoroMax3, NJ) as previously described.[30] Cells were cultured for 48 h treated with or without 1 µM RA to induce CD38 expression. $1x10^6$ cells were pelleted, washed twice with PBS, resuspended in 0.7 mL PBS, and treated with 100 µM NGD^+ for 1 h with constant rotation at 37°C. Cells were then pelleted and the supernatant was analysed for cGDPR. Readings at 300 nm excitation/410 nm emission were used to measure cGDPR content.

3.3-11 Statistics

Statistics were analysed using GraphPad Prism version 7.02. One-way and two-way analysis of variance, as appropriate, with Tukey's multiple comparisons test was used to determine significance. Error bars indicate mean ± standard error of the mean (SEM).

3.4 Results

3.4-1 Design and synthesis of a small molecule dimerizer of CD38

To induce CD38 homodimerization, our approach was to synthesize a dimer of the small molecule arabinosyl-2'-fluoro-2'-deoxy NAD^+ (F-araNAD$^+$), a suicide substrate of CD38.[37,38] The synthesis involved conjugating two 6-alkyne-F-araNAD$^+$ molecules via a diazido linker using the copper-catalysed Huisgen 1,3-dipolar cycloaddition between the alkyne and the azide functional groups, commonly known as click chemistry (Figure 3.1*A*).[39] The synthesis of 6-alkyne-F-araNAD$^+$ followed a previous report, with the exception of the phosphate coupling reaction between 6-alkyne-AMP and a fluorinated nicotinamide mononucleotide, arabinosyl-2'-fluoro-2'-deoxy nicotinamide mononucleotide (F-araNMN).[40] The modified coupling involved preparing the magnesium salts of 6-alkyne-AMP and F-araNMN, followed by the coupling reaction using N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide in MOPS buffer (1.5 M, pH 7.4) to afford 6-alkyne-F-araNAD$^+$ (Figure 3.1*B*). The diazido linker **2** was made by coupling two molecules of 6-azidohexanoic acid and one molecule of the diamino compound, 1,2-Bis(2-aminoethoxy)ethane (Figure 3.1*B*). Finally, the diazido linker **2** was conjugated to 6-alkyne-F-araNAD$^+$ via click chemistry to obtain the desired dimeric F-araNAD$^+$ (dF-araNAD$^+$) (Figure 3.1*C*).

Figure 1

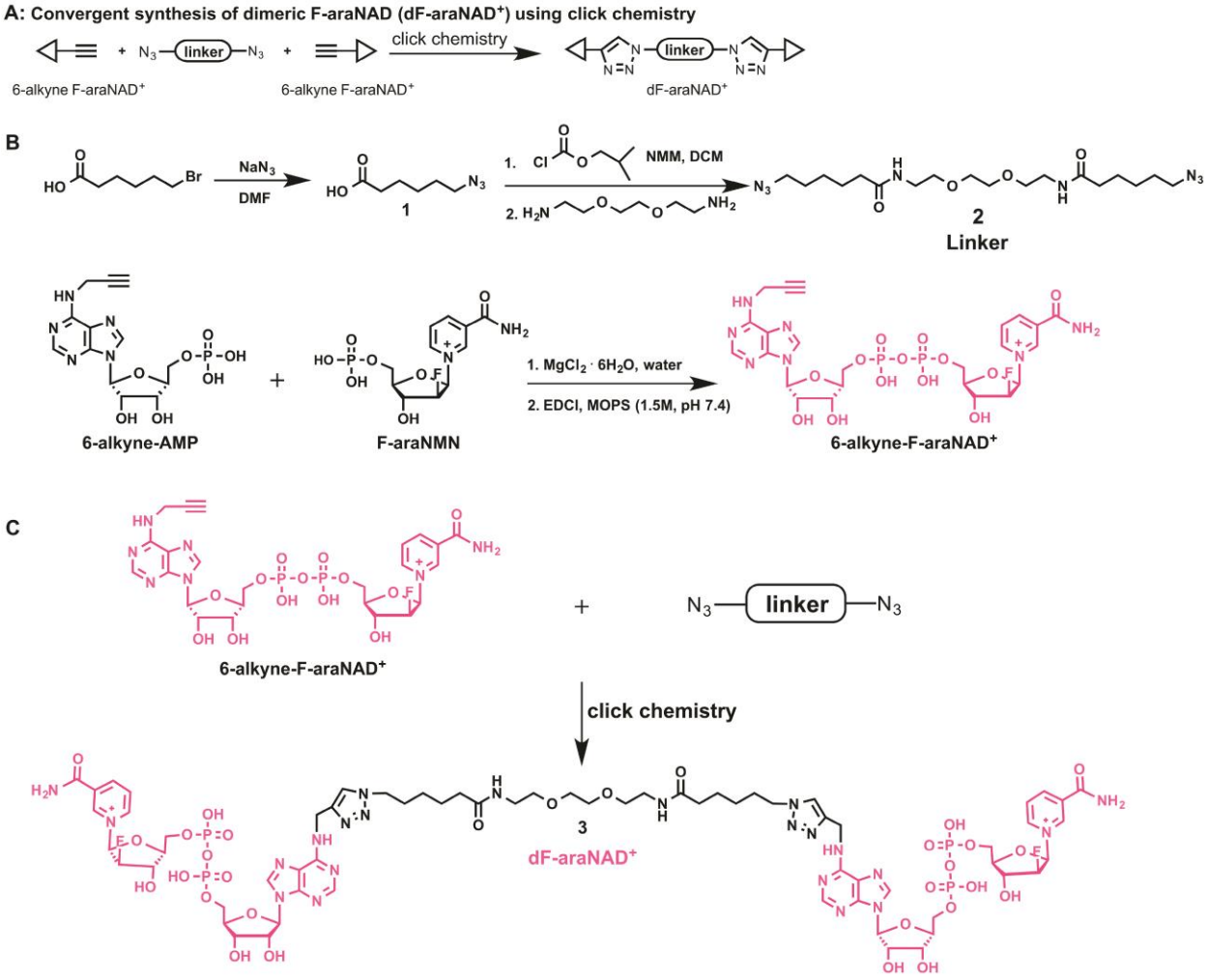


Figure 3.1: Synthesis of dimeric F-araNAD⁺ (dF-araNAD⁺). A: The strategy used for convergent synthesis of dF-araNAD⁺ using click chemistry. B: Synthesis of the linker and 6-alkyne-F-araNAD⁺ coupling reaction. C: Synthesis of dF-araNAD⁺ using click chemistry, showing molecular structure of the small molecule.

3.4-2 dF-araNAD$^+$ is able to induce covalent dimerization of CD38

We next examined whether dF-araNAD$^+$ was able to induce dimerization of CD38. We first tested whether dF-araNAD$^+$ can dimerize purified CD38 protein *in vitro*. As shown in Figure 3.2*A*, following a 30 min incubation period, dF-araNAD$^+$ was able to induce a degree of dimerization of purified CD38.

We then used FRET to detect CD38 dimerization in HL-60 cells.[41] We directly conjugated monoclonal antibodies against CD38 with either Alexa Fluor 488 or 594 and, after determining that treatment with either F-araNAD$^+$ or dF-araNAD$^+$ did not alter CD38 expression (Fig. 2*B*), we performed the FRET measurement via flow cytometry (Figure 3.2*C*). Untreated samples displayed background levels of fluorescence in the FRET channel. Samples with no CD38 antibodies are included to show that F-araNAD$^+$ and dF-araNAD$^+$ do not have background fluorescence. RA treatment induced CD38 expression and the acceptor fluorescence mean increased. When F-araNAD$^+$ was added, there was no effect on the acceptor fluorescence. When dF-araNAD$^+$ was added, however, the acceptor fluorescence mean increased. Therefore, in the presence of dF-araNAD$^+$, the donor and acceptor fluors were closer, supporting that dF-araNAD$^+$ increased the dimerization of CD38. Thus, while some CD38 homodimerization may be present after RA treatment alone, treatment with dF-araNAD$^+$ increases the degree of CD38 homodimerization. This indicates that not all available CD38 was homodimerized before addition of dF-araNAD$^+$.

Since dF-araNAD$^+$ functions via covalent interactions, we also assessed and confirmed CD38 dimerization in HL-60 cells via western blot.[37,38,40] HL-60 cells that had been treated with RA to induce CD38 expression were treated with dF-araNAD$^+$ at 8 h or 23 h and

harvested at 24 h. In both cases, a band corresponding to twice the molecular weight of CD38 was present in the samples treated with RA and dF-araNAD$^+$ (Figures 3.2*A* and 3.2*D*). These results were consistent with those of the purified CD38 and FRET experiments, indicating that dF-araNAD$^+$ induced dimerization of CD38.

Figure 2

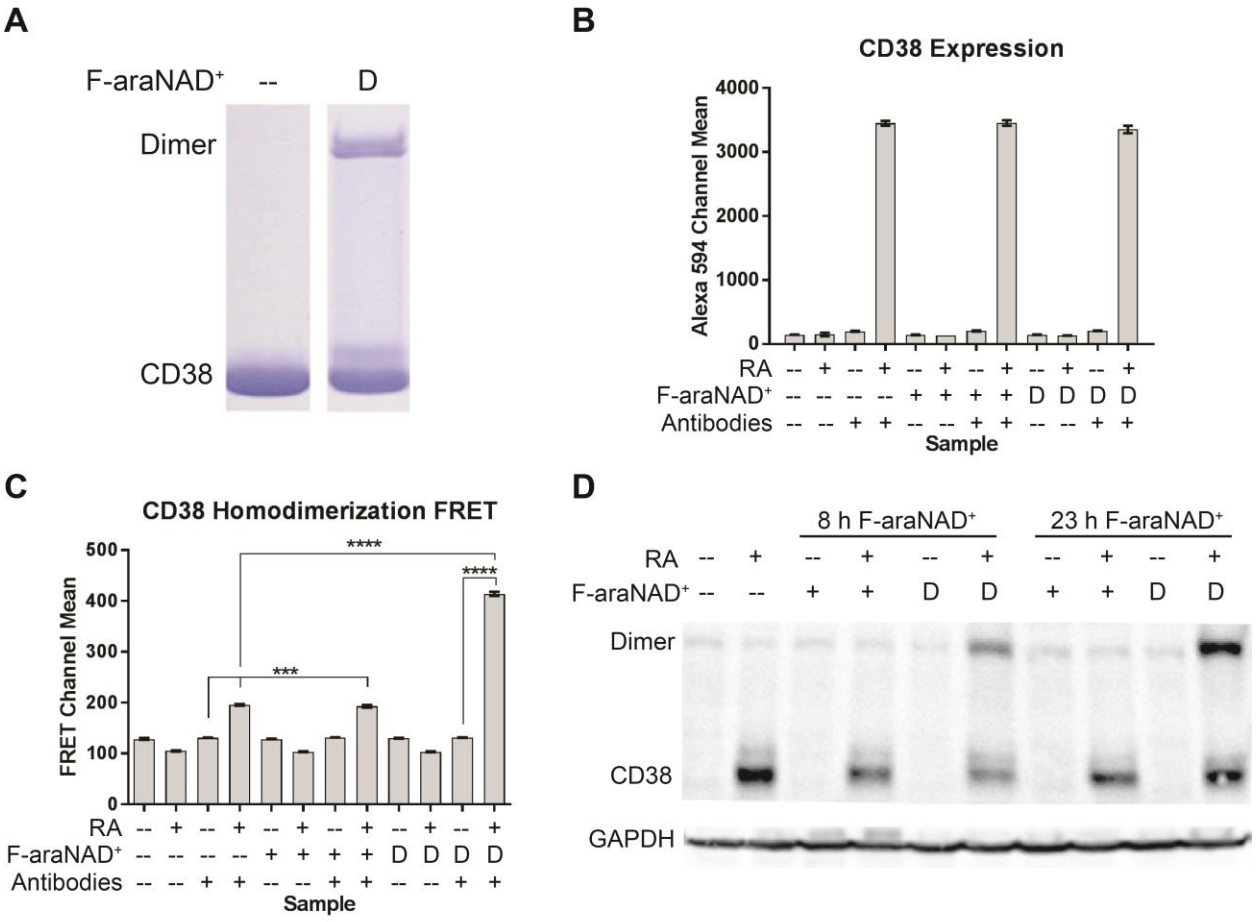

A
F-araNAD+ -- D
Dimer
CD38
B
CD38 Expression
Alexa 594 Channel Mean
4000
3000
2000
1000
0
RA -- + -- + -- + -- + -- + -- +
F-araNAD+ -- -- -- -- + + + + D D D D
Antibodies -- -- + + -- -- + + -- -- + +
Sample
C
CD38 Homodimerization FRET
FRET Channel Mean
500
400
300
200
100
0
****
****
***
RA -- + -- + -- + -- + -- + -- +
F-araNAD+ -- -- -- -- + + + + D D D D
Antibodies -- -- + + -- -- + + -- -- + +
Sample
D
8 h F-araNAD+
23 h F-araNAD+
RA -- + -- + -- + -- + -- +
F-araNAD+ -- -- + + D D + + D D
Dimer
CD38
GAPDH

Figure 3.2: dF-araNAD$^+$ induces CD38 homodimerization. A: *In vitro* labelling of purified CD38. 5 µM purified CD38 was treated with dF-araNAD$^+$ for 30 min as indicated. Reaction mixtures were resolved by SDS-PAGE and stained with Coomassie blue. B: Mean fluorescence of HL-60 cells stained with Alexa Fluor 594-conjugated CD38 antibody (n=3). HL-60 cells were cultured for 24 h with 1 µM RA as indicated. At 23 h, cells were treated with 1 µM F-araNAD$^+$ (+) or dF-araNAD$^+$ (D) as indicated. 1x10^6 cells were put in PBS with Alexa Fluor 594-conjugated CD38 antibody as indicated and analysed by flow cytometry. Error bars indicate standard error of the mean (SEM). C: FRET means (n=3). HL-60 cells were cultured for 24 h with 1 µM RA as indicated. At 23 h, cells were treated with 1 µM F-araNAD$^+$ (+) or dF-araNAD$^+$ (D) as indicated. 1x10^6 cells were put in PBS with Alexa Fluor 488- and 594-conjugated CD38 antibodies as indicated and analysed by flow cytometry. Error bars indicate SEM. ***, $p<0.001$; ****, $p<0.0001$. Two-way analysis of variance (ANOVA) using Tukey's multiple comparisons test was used to determine significance. D: Western blot showing covalent dimerization of CD38 when treated with dF-araNAD$^+$. HL-60 cells were cultured with RA and treated with F-araNAD$^+$ or dF-araNAD$^+$ at 8 or 23 h as indicated and lysate was collected at 24 h. 25 µg of lysate per lane was run. Membrane images for each protein are cropped to show only the bands of interest.

3.4-3 dF-araNAD$^+$ does not affect RA-induced differentiation

In order to assess the effects of CD38 dimerization on RA-induced differentiation, we analysed several myeloid lineage differentiation markers over the course of a 72 h treatment. Since a previous report demonstrated that CD38 catalytic activity has no influence on RA-induced differentiation, we were not concerned about blocking the active site.[30] We measured CD38 surface expression at 24 h and cell density (i.e., population growth), CD11b surface expression, and G_1/G_0 cell cycle arrest every 24 h. HL-60 cell cultures were initiated with or without RA at 0 h, and F-araNAD$^+$ or dF-araNAD$^+$ was added to flasks as indicated at 8 h or 23 h when CD38 expression was significantly induced. This represented testing for both early and late potential dimerization effects.

Expression levels of the differentiation markers varied as a function of RA, but not F-araNAD$^+$ or dF-araNAD$^+$, treatment (Figure 3.3). As expected, with RA treatment, CD11b and CD38 expression increased, the proportion of cells in G_1/G_0 increased, and cell population growth was retarded compared to control cells not treated with RA. Addition of F-araNAD$^+$ or dF-araNAD$^+$ at either time point, however, had no effect on the induced expression of any of these markers.

Figure 3

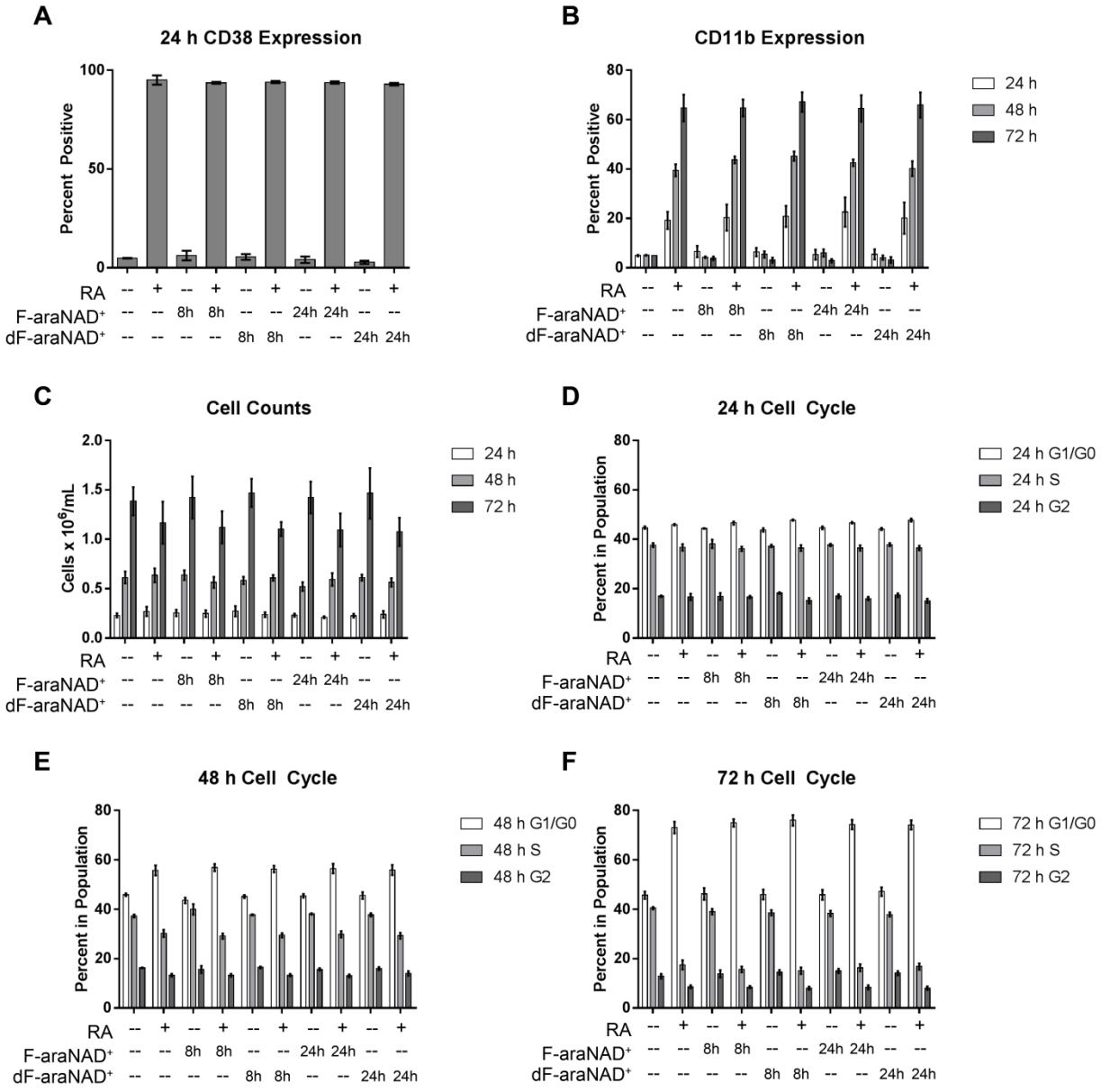

A
24 h CD38 Expression
Percent Positive
100
50
0
RA -- + -- + -- + -- + -- +
F-araNAD+ -- -- 8h 8h -- -- 24h 24h -- --
dF-araNAD+ -- -- -- -- 8h 8h -- -- 24h 24h
B
CD11b Expression
Percent Positive
80
60
40
20
0
24 h
48 h
72 h
RA -- + -- + -- + -- + -- +
F-araNAD+ -- -- 8h 8h -- -- 24h 24h -- --
dF-araNAD+ -- -- -- -- 8h 8h -- -- 24h 24h
C
Cell Counts
Cells x 10^6/mL
2.0
1.5
1.0
0.5
0.0
24 h
48 h
72 h
RA -- + -- + -- + -- + -- +
F-araNAD+ -- -- 8h 8h -- -- 24h 24h -- --
dF-araNAD+ -- -- -- -- 8h 8h -- -- 24h 24h
D
24 h Cell Cycle
Percent in Population
80
60
40
20
0
24 h G1/G0
24 h S
24 h G2
RA -- + -- + -- + -- + -- +
F-araNAD+ -- -- 8h 8h -- -- 24h 24h -- --
dF-araNAD+ -- -- -- -- 8h 8h -- -- 24h 24h
E
48 h Cell Cycle
Percent in Population
80
60
40
20
0
48 h G1/G0
48 h S
48 h G2
RA -- + -- + -- + -- + -- +
F-araNAD+ -- -- 8h 8h -- -- 24h 24h -- --
dF-araNAD+ -- -- -- -- 8h 8h -- -- 24h 24h
F
72 h Cell Cycle
Percent in Population
80
60
40
20
0
72 h G1/G0
72 h S
72 h G2
RA -- + -- + -- + -- + -- +
F-araNAD+ -- -- 8h 8h -- -- 24h 24h -- --
dF-araNAD+ -- -- -- -- 8h 8h -- -- 24h 24h

Figure 3.3: Induction of CD38 homodimerization does not phenotypically affect RA-induced differentiation. A: HL-60 cells were cultured for 24 h with 1 μM RA as indicated and membrane CD38 was analysed using flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. 1 μM F-araNAD$^+$ or dF-araNAD$^+$ was added at 8 or 24 h as indicated. B: Membrane CD11b was analysed using flow cytometry at 24, 48, and 72 h. C: Cell density was measured at 24, 48, and 72 h using a haemocytometer and 0.2% Trypan Blue exclusion staining. D-F: Cell cycle distribution, showing the percentage of cells in G_1/G_0, S, G_2/M, was analysed using flow cytometry with propidium iodide staining at 24, 48, and 72 h. Error bars indicate SEM.

3.4-4 Characterization of CRISPR/Cas9-mediated CD38 disruption

Using the CRISPR/Cas9 system, we generated three CD38-targeted cell lines, CRISPR 1, 2, and 3, to test whether CD38 is required for RA-induced differentiation. Using the E-CRISP design tools, we targeted sites as early as possible along the gene to perform knockouts.[32]

To ensure successful disruption, CD38 levels were measured by both flow cytometry and by western blot following 48 h treatment with RA. We detected no CD38 expression in any of the pooled cell lines (Figures 3.4*B* and 3.5*A*). We further characterized CD38 expression in the CRISPR-derived cell lines using RT-PCR (Figure 3.4*A*). Comparing wild-type CD38 with the truncated forms, CRISPR 1 and 3 both lack nearly the entire extracellular domain but retain the cytoplasmic and transmembrane domains (Figure 3.4*A*). CRISPR 1 retains the N-terminal 49 amino acids before a substitution at position 50 and a premature stop codon. CRISPR 3 has a CD38 product of 62 amino acids before a premature stop codon, with the first 45 amino acids of wild-type CD38 conserved (Figure 3.4*A*.). CRISPR 2 is a mixed population, as we saw two distinct bands after performing RT-PCR. Sequencing both bands, however, revealed that the CD38 truncations were similar – both lacked the vast majority of the protein, with only the first six or seven amino acids of CD38 left (Figure 3.4*A*.). Both had premature stop codons after 15 amino acids (Figure 3.4*A*).

The extracellular domain of CD38 contains its enzymatically active domain, and since we largely deleted this, we anticipated a loss of RA-induced enzymatic activity with the CRISPR mutants. We confirmed this using the nicotinamide guanine dinucleotide (NGD^+) assay (Figure 3.4*C*), which detects ADP-ribosyl cyclase activity. While CD38 is a major

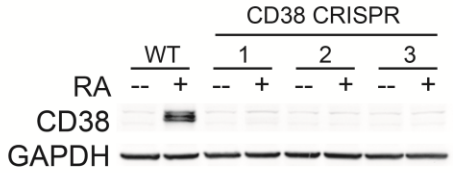
source of this activity, CD157 also possesses this catalytic activity.[42] HL-60 cells normally express both CD38 and CD157 at very low levels, but they are upregulated following RA treatment. We calculated the fold increase in cyclic GDP ribose (cGDPR) production from RA-treated to untreated cells for each mutant line and normalized to wild-type HL-60 levels. In each of the CRISPR-derived lines, RA-induced production of cGDPR was significantly reduced, further confirming the disruption of CD38.

Figure 4

A

	Cytoplasmic Domain	Transmembrane Domain	Extracellular Domain
CD38 WT	MANCEFSPVSGDKPCCRLSRR	AQLCLGVSILVLILVVVLAVV	VPRWRQQWSGPGTTKRFPETV
CRISPR 1	MANCEFSPVSGDKPCCRLSRR	AQLCLGVSILVLILVVVLAVV	VPRWRQQR-
CRISPR 3	MANCEFSPVSGDKPCCRLSRR	AQLCLGVSILVLILVVVLAVV	VPRHHQALSRDRPGAMRQVH-
CRISPR 2 lower	MANCEFSVLASVSWS-		
CRISPR 2 upper	MANCEFRGQTLLPAL-		

B



C

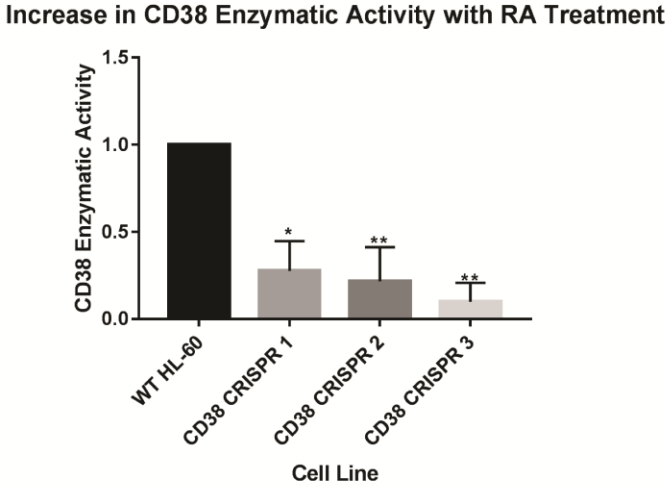


Figure 3.4: Disruption of CD38 using the CRISPR/Cas9 system. A: Sequence alignment comparing the CD38 amino acid sequence of each CRISPR-derived cell line, up to the first stop codon, to wild-type CD38. Highlight denotes sequence matching wild-type CD38. The CRISPR 2 line is a mixed population expressing two different CD38 truncations. B: Western blot of CD38. Wild-type and CRISPR-derived cell lines were treated with 1 μM RA as indicated for 48 h and whole cell lysate was collected. 25 μg of lysate per lane was run. Membrane images for each protein are cropped to show only the band of interest. C: CD38 enzymatic activity assay. Wild-type and CRISPR cell lines were cultured for 48 h with 1 μM RA as indicated to stimulate CD38 expression and evaluated for cGDPR production via an NGD^+ assay (n=4). The difference in fold increase between RA treated and untreated samples was taken for each cell line and normalized to a control value of 1 in wild-type cells. Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$. One-way ANOVA using Tukey's multiple comparisons test was used to determine significance. The raw data NGD^+ catabolism curves are shown in Supplementary Information.

3.4-5 CD38 disruption does not affect RA-induced differentiation

We evaluated the effects of CD38 disruption on RA-induced differentiation as we did with induced CD38 dimerization. We measured cell density, CD11b, CD38, and G_1/G_0 cell cycle arrest over the course of a 72 h RA treatment. For both wild-type and CRISPR-derived cell lines, RA treatment induced growth retardation, upregulation of CD38 and CD11b expression, and G_1/G_0 accumulation (Figure 3.5). The responses of the CRISPR-derived knockouts were not significantly different from wild-type cells except for the CRISPR 2 cell line, which displayed increased levels of CD11b at 48 and 72 h after RA treatment (Figure 3.5*B*).

Figure 5

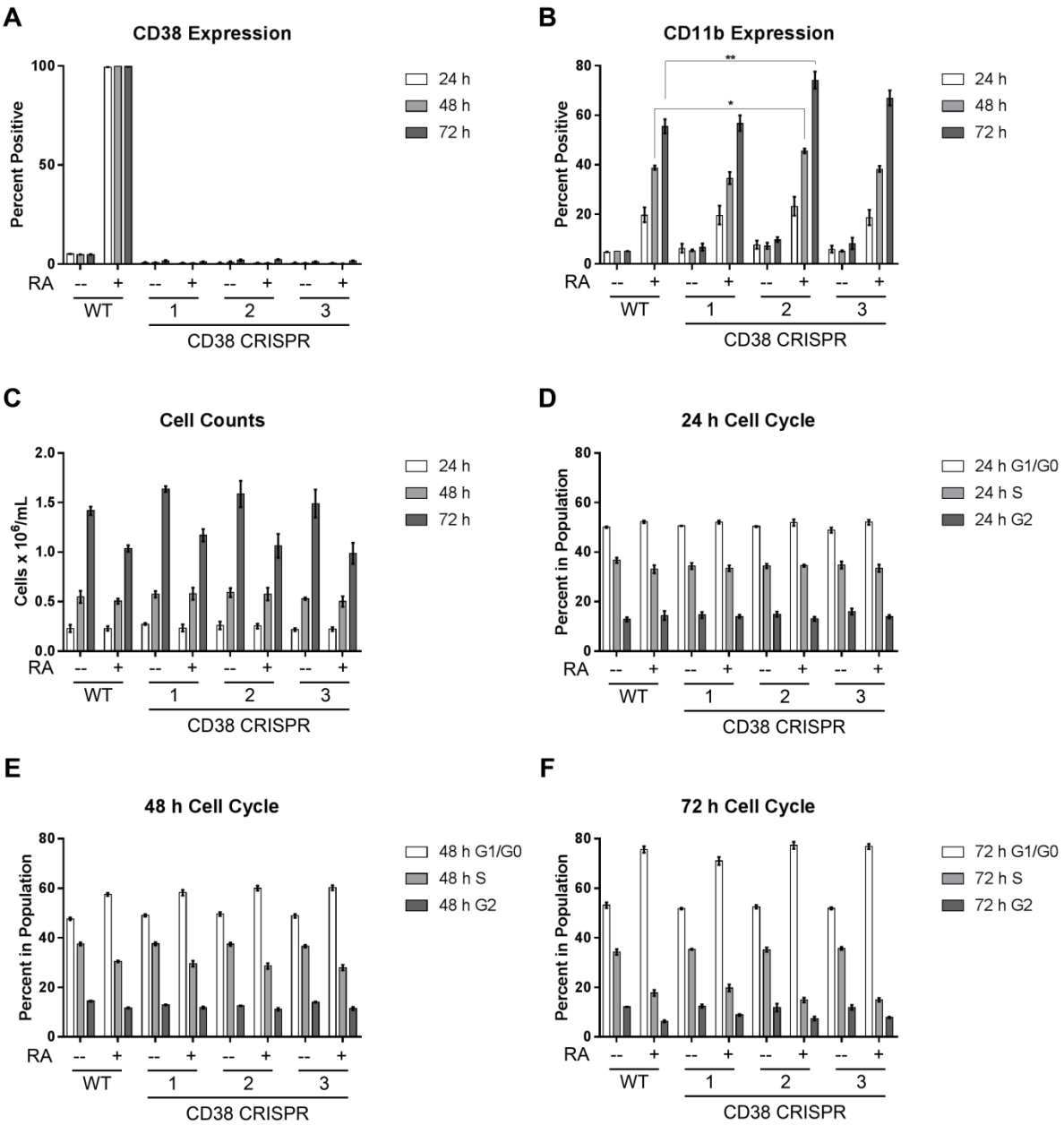

A
CD38 Expression
Percent Positive
100
50
0
RA
-- + -- + -- + -- +
WT
1
2
3
CD38 CRISPR
24 h
48 h
72 h
B
CD11b Expression
Percent Positive
80
60
40
20
0
**
*
RA
-- + -- + -- + -- +
WT
1
2
3
CD38 CRISPR
24 h
48 h
72 h
C
Cell Counts
Cells x 10^6/mL
2.0
1.5
1.0
0.5
0.0
RA
-- + -- + -- + -- +
WT
1
2
3
CD38 CRISPR
24 h
48 h
72 h
D
24 h Cell Cycle
Percent in Population
80
60
40
20
0
RA
-- + -- + -- + -- +
WT
1
2
3
CD38 CRISPR
24 h G1/G0
24 h S
24 h G2
E
48 h Cell Cycle
Percent in Population
80
60
40
20
0
RA
-- + -- + -- + -- +
WT
1
2
3
CD38 CRISPR
48 h G1/G0
48 h S
48 h G2
F
72 h Cell Cycle
Percent in Population
80
60
40
20
0
RA
-- + -- + -- + -- +
WT
1
2
3
CD38 CRISPR
72 h G1/G0
72 h S
72 h G2

Figure 3.5: CRISPR/Cas9-mediated disruption of CD38 does not phenotypically affect RA-induced differentiation. A: Wild-type and CRISPR-derived cell lines were cultured for 72 h with 1 μM RA as indicated and membrane CD38 expression was evaluated by flow cytometry at 24, 48, and 72 h. Gating to discriminate positive cells was set to exclude 95% of untreated wild-type cells. B: Membrane CD11b expression was analysed at 24, 48, and 72 h using flow cytometry. *, $p<0.05$; **, $p<0.01$. Two-way ANOVA using Tukey's multiple comparisons test was used to determine significance. C: Cell density was measured at 24, 48, and 72 h using a haemocytometer and 0.2% Trypan Blue exclusion staining. D-F: Cell cycle distribution was analysed using flow cytometry with propidium iodide staining at 24, 48, and 72 h. Error bars indicate SEM.

3.4-6 CD38 disruption does not affect prominent signalling proteins associated with RA-induced differentiation

Since an ensemble of signalling molecules propels differentiation, we determined whether any RA-response associated signalling proteins were affected by loss of the majority of CD38. c-Cbl, Lyn, Slp-76, and Vav1, which have been shown to interact with CD38, were of particular interest.[19–21] We treated wild-type HL-60s, as well as the CRISPR-derived cell lines, with or without RA for 48 h, collected lysates, and analysed them by western blot (Figure 3.6). For all the proteins we analysed, we observed that the RA-induced effects were similar for wild-type and CRISPR-derived cells (Figure 3.6). This corroborates the previously described results for phenotypic shift of the cells. Levels of the signalling proteins Lyn, c-Cbl, Slp-76, Vav1, and phospho-MEK increased similarly with RA treatment for wild-type and CRISPR-derived lines (Figure 3.6). RA treatment induced expression of a component of the NAD^+ phosphatase oxidase complex, $p47^{phox}$, in all cell lines as well (Figure 3.6). MEK and ERK levels remained stable (Figure 3.6).

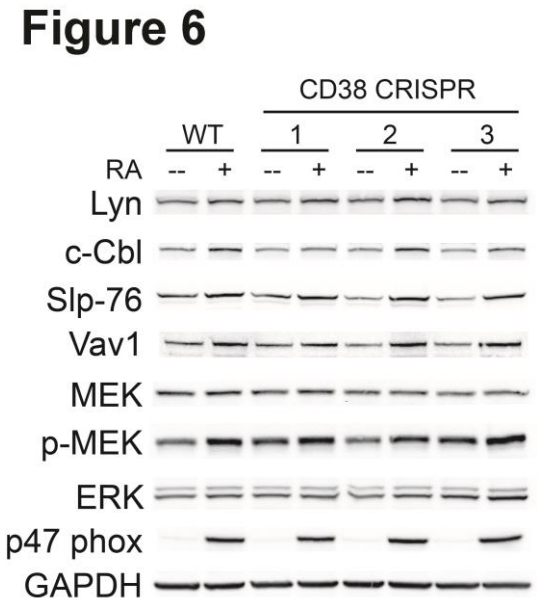


Figure 3.6: CRISPR/Cas9-mediated disruption of CD38 does not affect signalling proteins known to be involved in RA-induced differentiation. Wild-type and CD38 CRISPR cell lines were cultured for 48 h with 1 μM RA as indicated and whole cell lysate was collected. 25 μg of lysate per lane was run. Western blots of PAGE-resolved lysates from wild-type cells and CRISPR 1, 2, and 3 cell lines were probed for the indicated proteins, where GAPDH is a loading control. Membrane images for each protein are cropped to show only the band of interest.

3.5 Discussion

While a previous report indicated that CD38 is found as homodimers on the cell surface of murine B lymphocytes, our results suggest that dF-araNAD$^+$ is able to induce an additional degree of CD38 homodimerization in HL-60 cells.[31] This increased CD38 homodimerization did not affect RA-induced differentiation. We must note, however, that there are several alternative explanations for why dF-araNAD$^+$ did not affect RA-induced differentiation. It is possible that the induced CD38 dimer is not in the correct orientation to initiate downstream signalling or that high-order oligomerization other than dimerization is needed.

Additionally, we do not know whether a particular degree of dimerization is required for signalling. One of the best studied transmembrane receptors, epidermal growth factor receptor (EGFR), is typically found in a 3:7 ratio of dimer to monomer under resting conditions and a 1:1 ratio when its cognate ligand, EGF, is bound.[43] The western blot of relative abundance of CD38 monomer and dimers, although not quantitative, appears consistent with this range of receptor dimerization. *In vitro* dimerization of EGFR with EGF and activation of EGFR tyrosine kinase activity are coincident events, but transmembrane receptor dimer formation and downstream signalling are not always coupled.[44–46] It is experimentally intractable to test every possibility such as the dimer to monomer ratios, relation between dimer formation and downstream signalling, required orientation, and potential high-order oligomerization for CD38. Thus, we took a simpler alternative approach to study the role of CD38 in RA-induced differentiation: We genetically disrupted endogenous CD38 using the CRISPR/Cas9 system to probe for effects of losing the protein.

Results from the CRISPR/Cas9-mediated CD38 truncations indicate that the extracellular domain of CD38 is not necessary for RA-induced differentiation. CRISPR 1 and CRISPR 3 possess only seven and three amino acids, respectively, of the extracellular domain of CD38, while CRISPR 2 has none, yet the phenotypic response to RA treatment is largely unaltered. Because the enzymatic activity of CD38 is dependent on the extracellular domain, specifically E226, we also conclude that this enzymatic activity is not necessary for RA-induced differentiation, consistent with previously reported results.[30]

CRISPR 2 is a mixed population expressing two different short peptides of CD38, both of which are 15 amino acids long, with the first six or seven amino acids conserved. Phenotypic markers for RA-induced differentiation in the CRISPR 2 line were equal to or, in the case of CD11b, better than wild-type HL-60 cells. The transmembrane portion and much of the cytoplasmic domain are thus also not required for RA-induced differentiation. We cannot rule out the importance of the remaining short peptide; however, the weight of the evidence suggests that CD38 is not necessary for RA-induced myeloid differentiation. This conclusion can be integrated with that of an earlier report showing that siRNA- and morpholino-mediated knockdown of CD38 compromised RA-induced differentiation of HL-60 cells.[18] That report tested differentiation using only one assay, nitroblue tetrazolium reduction, a marker for inducible reactive oxidative metabolism. Occurrence of mature myeloid cells is commonly measured by either the CD11b cell surface marker or by functional differentiation evidenced by 12-*O*-tetradecanoylphorbol-13-acetate-inducible superoxide production. It is also betrayed by occurrence of G_1/G_0 arrest. While Munshi *et al.* measured superoxide production, which is a functional marker for mature cells, we measured CD11b, which is a widely accepted surface marker for mature cells, as well as

G_0 arrest and expression of $p47^{phox}$, a signature component of the superoxide production machinery. Evidenced by the cell surface and cell cycle indicators, there was cell maturation and cell cycle arrest following treatment of the CRISPR-derived cell lines with RA. Induced expression of $p47^{phox}$ was also not compromised. Synthesizing our data and theirs, it is possible that while some aspect of the NADPH machinery generating superoxide is CD38 dependent, the expression of the mature cell surface marker is not, nor is cell cycle arrest and nor is the induced expression of $p47^{phox}$. It is potentially also noteworthy that crippling CD38 may affect NAD^+ metabolism so that it adversely affects the function of NADPH machine and ergo superoxide production. Without being able to rule this out, superoxide production may not be a reliable marker of differentiation when NAD^+ metabolism is subject to manipulation. Hence, unless a 6-amino acid fragment of the cytoplasmic tail can account for any differentiation-driving action of the entire CD38 receptor, it appears that CD38 is not necessary for the signalling or differentiation attributed to RA.

In summary, our data show that despite the evidence suggesting that CD38 is a driver of RA-induced differentiation, neither induction of dimerization nor deletion of the vast majority of the protein affected the differentiation process. It appears that CD38 is ergo not necessary for RA to induce differentiation, even though enhancing its expression can enhance signalling and differentiation. A full understanding of its function remains to be determined. Given that CD38 is upregulated so prominently and so early in response to RA treatment, it is possible that it may play an important role in the function of differentiated cells. HL-60 cells differentiate into neutrophil-like cells after RA treatment.

Consistent with this, CD38 knockout mice are known to have immune defects.[47] However, exactly how CD38 affects the function of immune cells remains to be elucidated.

REFERENCES

1. Deshpande, D. A. *et al.* CD38/cyclic ADP-ribose signaling: role in the regulation of calcium homeostasis in airway smooth muscle. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **288,** L773–L788 (2005).
2. Adebanjo, O. a *et al.* A new function for CD38/ADP-ribosyl cyclase in nuclear Ca2+ homeostasis. *Nat. Cell Biol.* **1,** 409–414 (1999).
3. Sun, L. *et al.* CD38/ADP-ribosyl cyclase: A new role in the regulation of osteoclastic bone resorption. *J. Cell Biol.* **146,** 1161–1171 (1999).
4. Kato, I. *et al.* CD38 disruption impairs glucose-induced increases in cyclic ADP-ribose, [Ca2+](i), and insulin secretion. *J. Biol. Chem.* **274,** 1869–1872 (1999).
5. Jin, D. *et al.* CD38 is critical for social behaviour by regulating oxytocin secretion. *Nature* **446,** 41–45 (2007).
6. Moreno-García, M. E. *et al.* CD38 signaling regulates B lymphocyte activation via a phospholipase C (PLC)-gamma 2-independent, protein kinase C, phosphatidylcholine-PLC, and phospholipase D-dependent signaling cascade. *J. Immunol.* **174,** 2687–2695 (2005).
7. Sandoval-Montes, C. & Santos-Argumedo, L. CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines. *J. Leukoc. Biol.* **77,** 513–521 (2005).
8. Deaglio, S. *et al.* Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J. Immunol.* **160,** 395–402 (1998).
9. Liu, Z. *et al.* Elevated relative fluorescence intensity of CD38 antigen expression on CD8+ T cells is a marker of poor prognosis in HIV infection: Results of 6 years of follow-up. *Commun. Clin. Cytom.* **26,** 1–7 (1996).
10. Ondoa, P. *et al.* Evaluation of HIV-1 p24 antigenemia and level of CD8+CD38+ T cells as surrogate markers of HIV-1 RNA viral load in HIV-1-infected patients in Dakar, Senegal. *J. Acquir. Immune Defic. Syndr.* **41,** 416–424 (2006).
11. D'Arena, G. *et al.* CD38 expression correlates with adverse biological features and predicts poor clinical outcome in B-cell chronic lymphocytic leukemia. *Leuk. Lymphoma* **42,** 109–114 (2001).
12. Keyhani, A. *et al.* Increased CD38 expression is associated with favorable prognosis in adult acute leukemia. *Leuk. Res.* **24,** 153–159 (2000).
13. Breitman, T. R., Selonick, S. E. & Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2936–2940 (1980).
14. Collins, S. J. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* **70,** 1233–1244 (1987).
15. Drach, J., Zhao, S., Malavasi, F. & Mehta, K. Rapid induction of CD38 antigen on myeloid leukemia cells by all trans-retinoic acid. *Biochem. Biophys. Res. Commun.* **195,** 545–550 (1993).
16. Drach, J. *et al.* Retinoic acid-induced expression of CD38 antigen in myeloid cells is mediated through retinoic acid receptor-alpha *Cancer Res.* **54,** 1746–1752 (1994).
17. Lamkin, T. J. *et al.* Retinoic acid-induced CD38 expression in HL-60 myeloblastic leukemia cells regulates cell differentiation or viability depending on expression

levels. *J. Cell. Biochem.* **97,** 1328–1338 (2006).
18. Munshi, C. B., Graeff, R. & Lee, H. C. Evidence for a causal role of CD38 expression in granulocytic differentiation of human HL-60 cells. *J. Biol. Chem.* **277,** 49453–49458 (2002).
19. Shen, M. & Yen, A. c-Cbl interacts with CD38 and promotes retinoic acid-induced differentiation and G0 arrest of human myeloblastic leukemia cells. *Cancer Res.* **68,** 8761–8769 (2008).
20. Congleton, J., Shen, M., MacDonald, R., Malavasi, F. & Yen, A. Phosphorylation of c-Cbl and p85 PI3K driven by all-trans retinoic acid and CD38 depends on Lyn kinase activity. *Cell. Signal.* **26,** 1589–1597 (2014).
21. Shen, M. & Yen, A. c-Cbl tyrosine kinase-binding domain mutant G306E abolishes the interaction of c-Cbl with CD38 and fails to promote retinoic acid-induced cell differentiation and G0 arrest. *J. Biol. Chem.* **284,** 25664–25677 (2009).
22. Congleton, J., MacDonald, R. & Yen, A. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. *Leukemia* **26,** 1180–1188 (2012).
23. Wang, J. & Yen, A. A MAPK-positive feedback mechanism for BLR1 signaling propels retinoic acid-triggered differentiation and cell cycle arrest. *J. Biol. Chem.* **283,** 4375–4386 (2008).
24. Yen, A., Roberson, M. S., Varvayanis, S. & Lee, A. T. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res.* **58,** 3163–3172 (1998).
25. Kontani, K. *et al.* Tyrosine phosphorylation of the c-cbl proto-oncogene product mediated by cell surface antigen CD38 in HL-60 cells. *J. Biol. Chem.* **271,** 1534–1537 (1996).
26. Zubiaur, M., Izquierdo, M., Terhorst, C., Malavasi, F. & Sancho, J. CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-ζ/ζ-associated protein-70 signaling pathways in Jurkat T lymphocytes. *J. Immunol.* **159,** 193–205 (1997).
27. Zubiaur, M. *et al.* CD38 is associated with lipid rafts and upon receptor stimulation leads to Akt/protein kinase B and Erk activation in the absence of the CD3-zeta immune receptor tyrosine-based activation motifs. *J. Biol. Chem.* **277,** 13-22 (2001).
28. Ausiello, C. M. *et al.* Secretion of IFN-gamma, IL-6, granulocyte-macrophage colony-stimulating factor and IL-10 cytokines after activation of human purified T lymphocytes upon CD38 ligation. *Cell. Immunol.* **173,** 192–197 (1996).
29. Ausiello, C. M. *et al.* Functional topography of discrete domains of human CD38. *Tissue Antigens* **56,** 539–547 (2000).
30. Congleton, J., Jiang, H., Malavasi, F., Lin, H. & Yen, A. ATRA-induced HL-60 myeloid leukemia cell differentiation depends on the CD38 cytosolic tail needed for membrane localization, but CD38 enzymatic activity is unnecessary. *Exp. Cell Res.* **317,** 910–919 (2011).
31. Moreno-García, M. E. *et al.* CD38 is expressed as noncovalently associated homodimers on the surface of murine B lymphocytes. *Eur. J. Biochem.* **271,** 1025–

1034 (2004).
32. Heigwer, F., Kerr, G. & Boutros, M. E-CRISP: fast CRISPR target site identification. *Nat. Methods* **11,** 122-123 (2014).
33. Munshi, C. *et al.* Identification of the enzymatic active site of CD38 by site-directed mutagenesis. *J. Biol. Chem.* **275,** 21566–21571 (2000).
34. Liu, Q. *et al.* Crystal structure of human CD38 extracellular domain. *Structure* **13,** 1331–1339 (2005).
35. Bunaciu, R. P. *et al.* 6-formylindolo(3,2-b)carbazole (FICZ) modulates the signalsome responsible for RA-induced differentiation of HL-60 myeloblastic leukemia cells. *PLoS One* **10,** e0135668 (2015).
36. Reiterer, G. & Yen, A. Platelet-derived growth factor receptor regulates myeloid and monocytic differentiation of HL-60 cells. *Cancer Res.* **67,** 7765–7772 (2007).
37. Sleath, P. R., Handlon, A. L. & Oppenheimer, N. J. Pyridine coenzyme analogs. 3. Synthesis of three NAD+ analogs containing a 2'-deoxy-2'-substituted nicotinamide arabinofuranosyl moiety. *J. Org. Chem.* **56,** 3608–3613 (1991).
38. Sauve, A. A. & Schramm, V. L. Mechanism-based inhibitors of CD38: A mammalian cyclic ADP-ribose synthetase. *Biochemistry* **41,** 8455–8463 (2002).
39. Kolb, H. C., Finn, M. G. & Sharpless, K. B. Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed.* **40,** 2004–2021 (2001).
40. Jiang, H. *et al.* Mechanism-based small molecule probes for labeling CD38 on live cells. *J. Am. Chem. Soc.* **131,** 1658–1659 (2009).
41. Förster, T. Intermolecular energy transference and fluorescence. *Ann. Phys.* **2,** 55–75 (1948).
42. Hussain, A. M. M., Lee, H. C. & Chang, C. F. Modulation of CD157 expression in multi-lineage myeloid differentiation of promyelocytic cell lines. *Eur. J. Cell Biol.* **79,** 697–706 (2000).
43. Kluba, M., Engelborghs, Y., Hofkens, J. & Mizuno, H. Inhibition of receptor dimerization as a novel negative feedback mechanism of EGFR signaling. *PLoS One* **10,** e0139971 (2015).
44. Sridharan, R., Connelly, S. M., Naider, F. & Dumont, M. E. Variable dependence of signaling output on agonist occupancy of Ste2p, a G protein coupled receptor in yeast. *J. Biol. Chem.* **291,** 24261-24279 (2016).
45. Canals, F. Signal transmission by epidermal growth factor receptor: coincidence of activation and dimerization. *Biochemistry* **31,** 4493–4501 (1992).
46. Yu, X., Sharma, K., Takahashi, T., Iwamoto, R. & Mekada, E. Ligand-independent dimer formation of epidermal growth factor receptor (EGFR) is a step separable from ligand-induced EGFR signaling. *Mol. Biol. Cell* **13,** 2547–2557 (2002).
47. Cockayne, D. a *et al.* Mice deficient for the ecto-nicotinamide adenine dinucleotide glycohydrolase CD38 exhibit altered humoral immune responses. *Blood* **92,** 1324–33 (1998).

Chapter 4 Foreword

This chapter represents a shift from pursuit of CD38 as a potential key driver of RA-induced differentiation to picking up on older studies on CXCR5 that were shelved as a result of the lack of availability of a reliable commercially available antibody against CXCR5. Data presented in this chapter was collected by Robert MacDonald and the chapter mirrors a manuscript written by Robert MacDonald and edited by Dr. Andrew Yen that has not yet been accepted for publication.

Chapter 4: CXCR5 overexpression in HL-60 cells enhances chemotaxis toward CXCL13 without anticipated interaction partners or enhanced MAPK signaling

4.1 Abstract

CXCR5 is a serpentine receptor implicated in controlling cell migration in lymphocytes and differentiation in leukocytes. It is known to cause MAPK pathway activation and has known membrane partners for signaling. CXCR5 is reportedly expressed in neutrophils following isolation, but its role in this cellular context is not known. Expression of CXCR5 mRNA has also been observed in HL-60 cells, a human acute myeloid leukemia line, following treatment with all-*trans* retinoic acid (RA), which induces differentiation toward a neutrophil-like state. Previous reports indicate that it is necessary for differentiation, as the process was crippled in CXCR5 knockout cells and enhanced in cells ectopically expressing it. Since CXCR5 has various membrane protein partners and potentially uses such classical dimerization effects to signal, we investigated whether CXCR5 signaling driving RA-induced differentiation depends on its association with such partners. Pursuing this, we generated HL-60 cells overexpressing the protein. We found that CXCR5 drove migration toward its ligand, CXCL13, and then probed for its interactions with several candidates, including three reported in other cellular contexts, using an established flow cytometry-based Förster resonance energy transfer method. Surprisingly, using wild-type and CXCR5-overexpressing HL-60 cells with or without 24 h RA treatment, we did not detect interactions with any of our candidates, including the previously reported partners. Additionally, we observed no significant changes in RA-induced differentiation; this may be a result of the stoichiometry of CXCR5 and partner receptors or CXCL13. The anticipated membrane partnerings were surprisingly apparently unnecessary for downstream CXCR5 signaling and RA-induced differentiation.

4.2 Introduction

CXCR5 was originally discovered as Burkitt's Lymphoma Receptor 1 (BLR1), in a subtractive hybridization screen of Epstein-Barr virus immortalized B cells subtracted from metastatic Burkitt's Lymphoma cells to find determinants of the metastatic phenotype.[1] It was also later found by differential display to also be differentially expressed in myeloid series cells as a function of differentiation.[2] CXCR5 is a 7-pass putative heterotrimeric G-protein coupled receptor capable of instigating mitogen-activated protein kinase (MAPK) pathway signaling.[1–3]

In lymphocytes, CXCR5 is essential for naïve B cell migration to follicles in lymph nodes and the spleen, where its ligand, CXCL13, is produced by stromal cells residing in these locations.[3–6] In addition to B cells, CXCR5 is also highly expressed on T follicular helper cells.[7,8] One possible explanation for these expression patterns is that CXCL13 helps to divide the B cell and T cell zones in the lymph nodes; the T follicular helper cells may interact with follicular B cells to activate them and form germinal centers.[9–11]

In myeloid series cells, CXCR5 mRNA expression has been detected in neutrophils following isolation and incubation for 3 h at 37^{o}C, though the consequences of expression of the protein was unclear.[12] It is potentially noteworthy that expression of CXCL13 is upregulated following a number of infections, such as with *Borrelia garinii*, *Chlamydia trachomatis*, and *Helicobacter pylori*, among others.[13–15] Additionally, CXCR5 is upregulated in HL-60 cells during all-*trans* retinoic acid (RA)-induced differentiation to neutrophil-like cells.[2,16] Hence in myeloid cells it has potential roles in response to infection and cell differentiation.

The HL-60 cell line, a human acute myeloid leukemia (AML) line, has been used as a model for all-*trans* retinoic acid (RA)-induced differentiation for several decades. The proteins and signaling pathways driving RA-induced differentiation, however, remain enigmatic. A previous report demonstrated that CXCR5 is necessary for the process, as bi-allelic knockout cripples differentiation.[17] Reports have also shown that ectopic expression of CXCR5 can enhance RA-induced differentiation, evidenced by increased activation of the MAPK signaling axis members c-Raf, MEK, and ERK; enhancement of G_1/G_0 cell cycle arrest; enhancement of the phenotypic marker of differentiation, CD11b; and enhancement of the functional marker of differentiation, inducible oxidative metabolism.[2,17,18]

Mechanistic details concerning signaling via CXCR5 remain unclear. Activation of CXCR5 by CXCL13 is characterized by chemotaxis toward CXCL13, a transient increase in intracellular calcium, and ERK signaling.[19] The steps directly linking CXCR5 to the generation of a MAPK signal, however, are unknown. In the context of several prostate cancer cell lines, CXCR5 has been shown to co-immunoprecipitate with CXCR4 as well as $G_{\alpha i2}$, $G_{\alpha q/11}$, $G_{\alpha 13}$, $G_{\beta 3}$, and $G_{\gamma 9}$.[20] CXCR5 has also been shown to homodimerize and to heterodimerize with EBI2 in HEK293T cells co-transfected to express the proteins.[21] The limited data thus suggest that CXCR5 might be following classical receptor activation paradigms regarding membrane and cytosolic partnering events.

We sought to fill in gaps in understanding of CXCR5 signaling by searching for interaction partners following the classical premise that receptor dimerization can be seminal to activation and signaling. We generated HL-60 transfectants overexpressing CXCR5 ($CXCR5^+$) and found that the protein was active, evidenced by chemotaxis toward

CXCL13. We then proceeded to probe for several candidate interaction partners found on the cell surface as well as two found in the cytosol. These interactions were probed using a previously established and validated flow cytometry-based Förster resonance energy transfer (FRET) method.[22] Our membrane protein candidates were CD11b, CD38, CXCR4, CXCR5 itself, and EBI2. CD11b and CD38 are both cell surface markers that are upregulated during RA-induced differentiation.[23,24] CD38, while not necessary for RA-induced differentiation, enhances the process when overexpressed.[22,25] CXCR4, CXCR5, and EBI2 were intended to be controls, as these interactions were previously reported in different cellular contexts.[20,21] Our two cytosolic candidates, c-Cbl and Lyn, have both been shown to play important roles in RA-induced differentiation.[26,27] Surprisingly, we were unable to detect interactions between CXCR5 and any of our candidates, including the three previously reported interactions found in different cellular contexts.[20,21] Unlike previous reports, the $CXCR5^{+}$ cells did not exhibit enhanced RA-induced differentiation or associated signaling, suggesting a stoichiometric dependence between CXCR5 and CXCL13 or interaction partners. We did not detect CXCR5 receptor interactions with other membrane partners amongst our ensemble of putative candidates that might contribute to receptor dimerization-mediated signaling in HL-60 cells. Having eliminated this obvious option for CXCR5-driven signaling, additional studies to elucidate the important role of CXCR5 in RA-induced differentiation and its potential role in neutrophil function are needed.

4.3 Materials and methods

4.3-1 Cell culture

HL-60 human myeloblastic leukemia cells, a generous gift of Dr. Robert Gallagher, were derived from the original patient isolates and maintained as previously described.[22] The line was certified and tested for mycoplasma by Bio-Synthesis, Lewisville, TX, USA in August 2017. Raji cells were a kind gift of Dr. Kristi Richards and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS (GE Healthcare, Chicago, IL) and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA). HL-60 cells transfected to overexpress CXCR5 ($CXCR5^+$) were maintained as HL-60 cells following selection described in section 2.3. RA was used at a final concentration of 1 μM.

4.3-2 Antibodies and reagents

Reagents, unless otherwise specified, were purchased from commercial suppliers in the highest purity available and used as supplied. PE-conjugated CD38, APC-conjugated CD11b, purified CD38, purified CD11b, CXCR4, and c-Raf antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ). Alexa Fluor 488-conjugated CXCR5, purified CXCR5, and EBI2 antibodies were purchased from BioLegend (San Diego, CA). Lyn antibody was purchased from Abcam (Cambridge, UK). c-Raf pS621 antibody was purchased from Sigma (St. Louis, MO). c-Cbl antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Total MEK1/2, phospho-MEK1/2 (S217/S221), total ERK1/2, phospho-ERK1/2 (T202/T404), and GAPDH antibodies were purchased from Cell Signaling Technologies (Danvers, MA).

4.3-3 Generation of stable transfectants

Human *CXCR5* cDNA (NCBI reference sequence NM_001716.4), originally obtained from Genecopoeia (Rockville, MD) in the EX-A1294-M55 vector, was cloned into pEF1a-IRES-Neo, a gift from Thomas Zwaka (plasmid #28019 from Addgene, Cambridge, MA).

CXCR5 was mobilized by PCR using PCR Supermix High Fidelity (Invitrogen, Carlsbad, CA) on a PTC-100 thermocycler (MJ Research, Waltham, MA). Primers to add N-terminal *Nhe*I and C-terminal *Not*I restriction sites were purchased from Integrated DNA Technologies (Coralville, IA) (*Nhe*I: 5'-TATGCCTAGCATGAACTACCCGCTAACGCTG-3', *Not*I: 5'-TATAGCGGCCGCCTAGAACGTGGTGAGAGAGGT-3'). The product was purified using a PureLink PCR Purification Kit (Invitrogen) following the manufacturer's protocol. *Nhe*I and *Not*I were purchased from New England Biolabs (NEB) (Ipswich, MA) and used according to the manufacturer's protocol to digest the purified PCR product and pEF1a-IRES-Neo. The cut insert and vector were then ligated using T4 DNA ligase (NEB), amplified in One Shot Top10 competent cells (Thermo Fisher Scientific, Waltham, MA), and purified using QIAprep Miniprep kits (Qiagen, Hilden, Germany), all according to manufacturer's instructions. Purified plasmid was sequenced to confirm appropriate *CXCR5* insertion.

To generate CXCR5$^+$, stable HL-60 transfectants overexpressing CXCR5, transfection was performed as previously described.[27] CXCR5$^+$ cells were selected for 2 weeks in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 1 mg/mL G418. CXCR5$^+$ cells were then purified via FACS using a Becton Dickinson FACS Aria III SORP (Becton Dickinson, Franklin Lakes, NJ) following immunostaining with an Alexa Fluor 488-conjugated anti-CXCR5 antibody (BioLegend, San Diego, CA) as previously described for CD11b.[22] Gating for sorting was set to include cells with fluorescence levels equal to or greater than the top 1% of stained wild type HL-60 cells. To validate stable transfection, CXCR5 levels in CXCR5$^+$, Raji (positive control), HL-60 (negative control), and 48 h RA-treated HL-60 cells were analyzed by flow cytometry.

4.3-4 Cell migration assay

To determine propensity of different cells to migrate toward CXCL13, the ligand for CXCR5, we used 6.5 mm Transwell inserts with 5.0 µM pore sizes in 24-well plates from Corning (Corning, NY) using a previously described migration assay.[28] Briefly, 600 µL of complete culture media with 0, 0.1, 0.5, or 2.5 µg/mL human recombinant CXCL13, purchased from BioLegend (San Diego, CA), was added to the bottom of each well. After 48 h of culture with (for HL-60) or without (for HL-60 and $CXCR5^+$) RA treatment, 1.5 x 10^5 cells were added to the inserts and the plate was incubated at 37°C with 5% CO_2 for 6 h. The inserts were then removed and wells were imaged using a BioRad ZOE fluorescent cell imager (Hercules, CA). Cells that had travelled through the membrane to the well were resuspended by pipetting, transferred to microcentrifuge tubes, and pelleted. Media was removed and cells were resuspended in 100 uL phosphate-buffered saline and counted using a hemocytometer. To determine percentage of cell migration for each sample, the total number of cells that had migrated to the wells was divided by the number of input cells and this proportion was multiplied by 100. We assumed negligible cell proliferation, as well as negligible differences in cell proliferation across samples, during the 6 h treatment period.

4.3-5 Förster resonance energy transfer (FRET) experiments

We probed for CXCR5 interaction partners using flow cytometry FRET using a method previously described and used to successfully detect CD38 homodimerization in HL-60 cells following treatment with RA and/or a synthetic CD38 linker.[22,29] Antibody conjugation for Alexa Fluor 546 conjugates (c-Cbl, CD11b, and Lyn) was performed as previously described.[22] Alexa Fluor 555 conjugates (CD38, CXCR4, CXCR5, and EBI2) were

created using an Alexa Fluor 555 antibody labeling kit from Thermo Fisher Scientific following the manufacturer's protocol. We chose to use these fluors for the acceptor as they are more sensitive than the Alexa Fluor 488/Alexa Fluor 594 pair according to the manufacturer. Although our experimental setup was largely performed according to the previously described method,[22] we adjusted our filter and mirror sets to accommodate Alexa Fluor 546 and 555, collecting through a 550 nm longpass dichroic mirror and a 585/42 nm filter. When running samples probing for interactions between CXCR5 and CD11b, CD38, CXCR4, CXCR5, and EBI2, live samples were run. When running samples probing for interactions between CXCR5 and c-Cbl and Lyn, two cytosolic proteins, cells were fixed prior to staining, as previously described.[30]

4.3-6 Phenotyping

Immunostaining for CD11b and CD38 was performed as previously described and analyzed using a Becton Dickinson LSR II flow cytometer.[22] Propidium iodide (PI) cell cycle analysis was performed as previously described.[22]

4.3-7 Western blot analysis

Western blots were performed as previously described.[22]

4.3-8 Statistics

Data were analyzed using GraphPad Prism version 7.02. One-way or two-way analysis of variance (ANOVA) was used to determine significance as appropriate. Error bars indicate mean ± standard deviation.

4.4 Results

4.4-1 Generation of stable transfectants overexpressing CXCR5

In order to probe for CXCR5 binding partners, we generated stable HL-60 transfectants overexpressing CXCR5 ($CXCR5^+$). $CXCR5^+$ and HL-60 cells were treated with RA for 48 h as indicated in Figure 1, a time when indications of overt differentiation evidenced by CD11b expression and G_1/G_0 arrest first become apparent.[23,31] HL-60 cells do not express CXCR5, and as shown in Figure 4.1, there is minimal background fluorescence; expression is mildly but significantly induced by RA treatment. The percentage of positive cells is much lower than that of the positive control, Raji cells, which are 100% positive.[2,32] $CXCR5^+$ cells, however, are 100% positive like Raji. $CXCR5^+$ cells have significantly greater CXCR5 per cell, measured by mean fluorescence per cell, than the Raji cells. The CXCR5 stable transfection thus achieved a high level of expression.

Figure 1

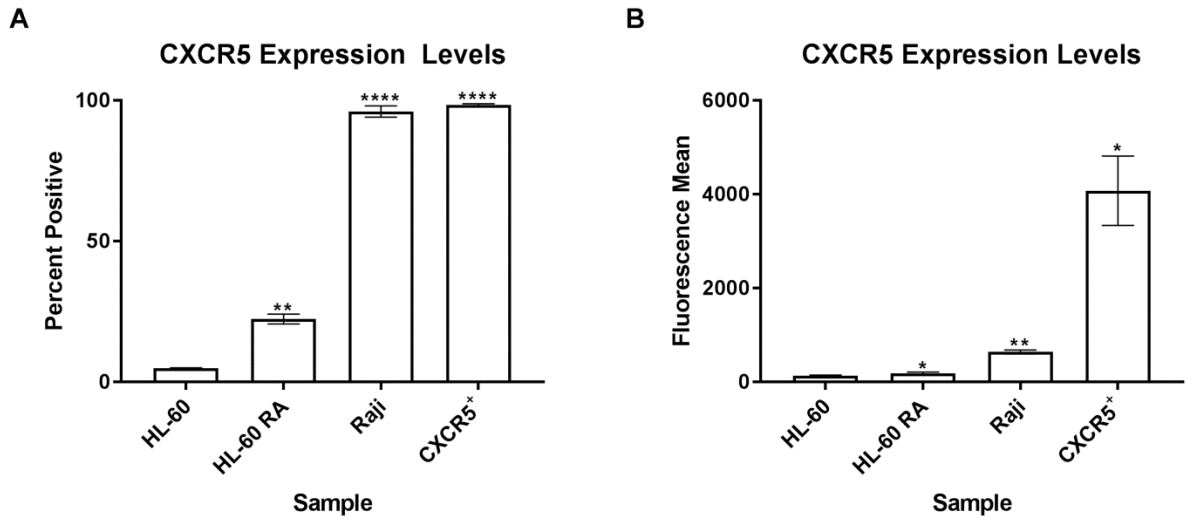


Figure 4.1: CXCR5$^+$ cells have high CXCR5 expression levels. A. CXCR5 expression levels in HL-60, RA-treated HL-60, Raji, and CXCR5$^+$ cells were measured by flow cytometry after a 48 h treatment period. Gating was set to exclude 95% of stained control cells (HL-60). n = 3. **, $p < 0.01$; ****, $p < 0.0001$ B. CXCR5 expression levels shown in terms of mean fluorescence levels. *, $p < 0.05$; **, $p < 0.01$. Error bars indicate standard deviation (SD).

4.4-2 CXCR5$^+$ cells migrate toward CXCL13

To determine that the CXCR5 expressed in the CXCR5$^+$ cell line was functional, we performed a cell migration assay testing for migration toward CXCL13, the ligand of CXCR5.[33] We compared migration of HL-60 cells, which do not express CXCR5, to that of RA-treated HL-60 cells (to induce CXCR5 expression) and CXCR5$^+$ cells. Following a previously reported standard experimental protocol for CXCL13-mediated migration, we measured the percentage of cells that migrated from Transwell inserts to wells below containing varying concentrations of CXCL13 in complete growth media.[28] As shown in Figure 4.2, we used concentrations between 0 and 2.5 µg/mL and did not observe chemotaxis in untreated HL-60 cells, which do not express CXCR5, over the 6 h incubation period. RA-treated HL-60 cells, in which RA has induced CXCR5 expression, migrated at low levels under all conditions, with increased migration at 0.5 µg/mL CXCL13. CXCR5$^+$ cells, which express high levels of CXCR5, did not migrate without ligand, migrated minimally at 0.1 µg/mL CXCL13, and had the highest degree of migration with 0.5 µg/mL CXCL13. For both RA-treated HL-60 cells and CXCR5$^+$ cells, migration decreased with 2.5 µg/mL CXCL13 compared to 0.5 µg/mL CXCL13. The ectopic CXCR5 ergo appears to be functional.

Figure 2

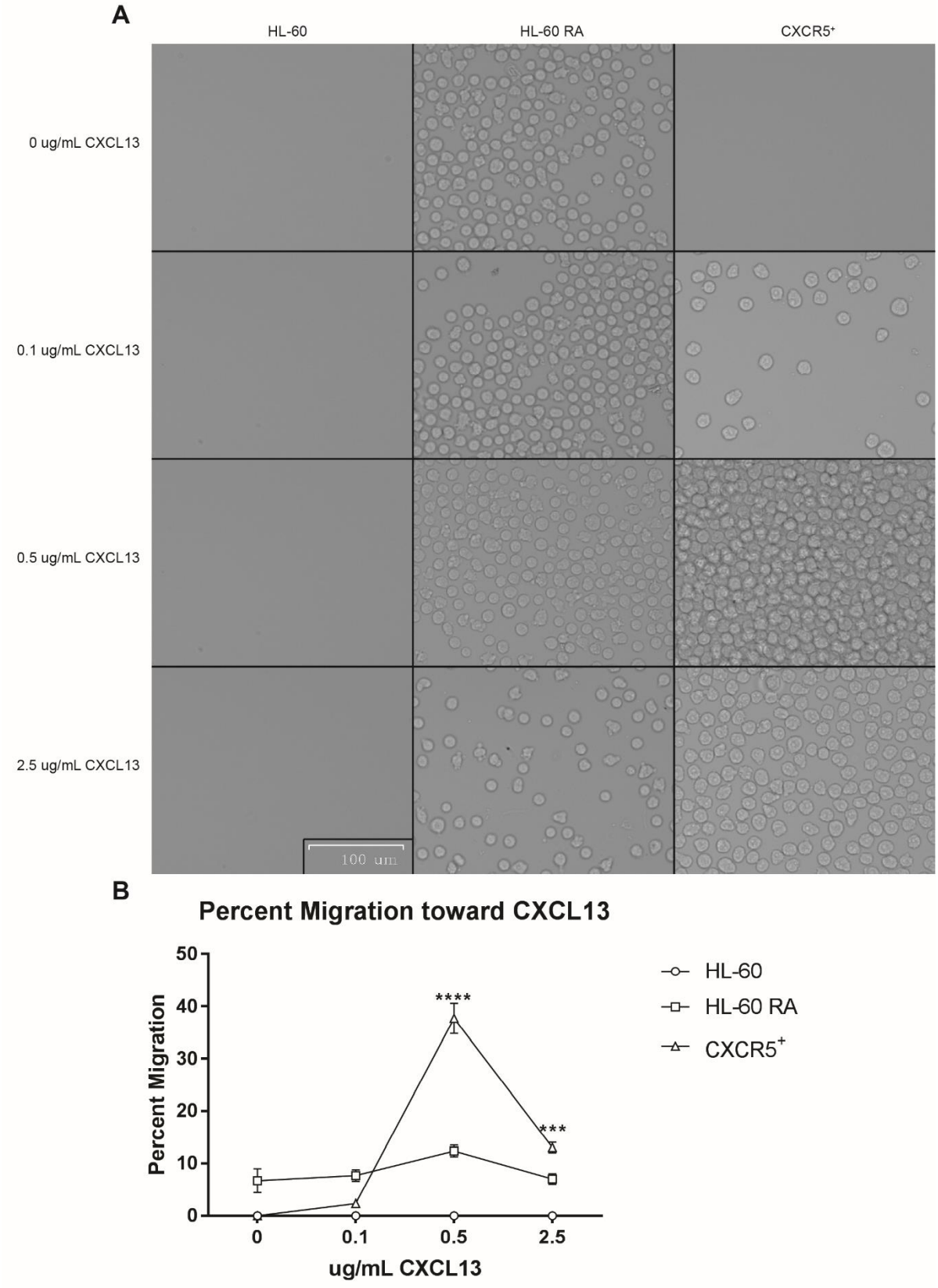

A
HL-60
HL-60 RA
CXCR5+
0 ug/mL CXCL13
0.1 ug/mL CXCL13
0.5 ug/mL CXCL13
2.5 ug/mL CXCL13
100 um
B
Percent Migration toward CXCL13
Percent Migration
50
40
30
20
10
0
****
***
0
0.1
0.5
2.5
ug/mL CXCL13
HL-60
HL-60 RA
CXCR5+

Figure 4.2: CXCR5$^+$ cells exhibit chemotaxis toward CXCL13. A. HL-60 and CXCR5$^+$ cells were cultured with (HL-60 RA) or without (HL-60, CXCR5$^+$) RA for 48 h and 1.5 x 10^5 cells were seeded into Transwell membranes over wells containing complete culture media supplemented with CXCL13 (0.1, 0.5, or 2.5 µg/mL) as indicated. After a 6 h incubation period, Transwell inserts were removed, wells were imaged, and cells from the wells were counted. Representative images of microscope fields are shown. B. Percent migration toward CXCL13 was calculated by dividing counts from the wells by the total number of cells seeded and then multiplying by 100. n = 3. Error bars indicate SD. ***, $p < 0.001$; ****, $p < 0.0001$.

4.4-3 No detectable FRET signal between CXCR5 and membrane protein candidates

We used a previously established and validated flow cytometry-based FRET assay to test whether CXCR5 interacts with CD11b, CD38, CXCR4, CXCR5, and EBI2. We chose this set of candidates based on their upregulation in RA-induced differentiation and their previously reported interactions in other cellular contexts.[20,21,23,24] As shown in Figure 4.3, HL-60 and $CXCR5^+$ cells were treated for 24 h with RA as indicated and FRET measurements were performed using the Alexa Fluor 488/Alexa Fluor 546 and Alexa Fluor 488/Alexa Fluor 555 pairs. We observed no enhancement of signal in the FRET channels above background, unstained levels; hence there was no FRET-measured dimerization of CXCR5 with any of the anticipated membrane partners.

Figure 3

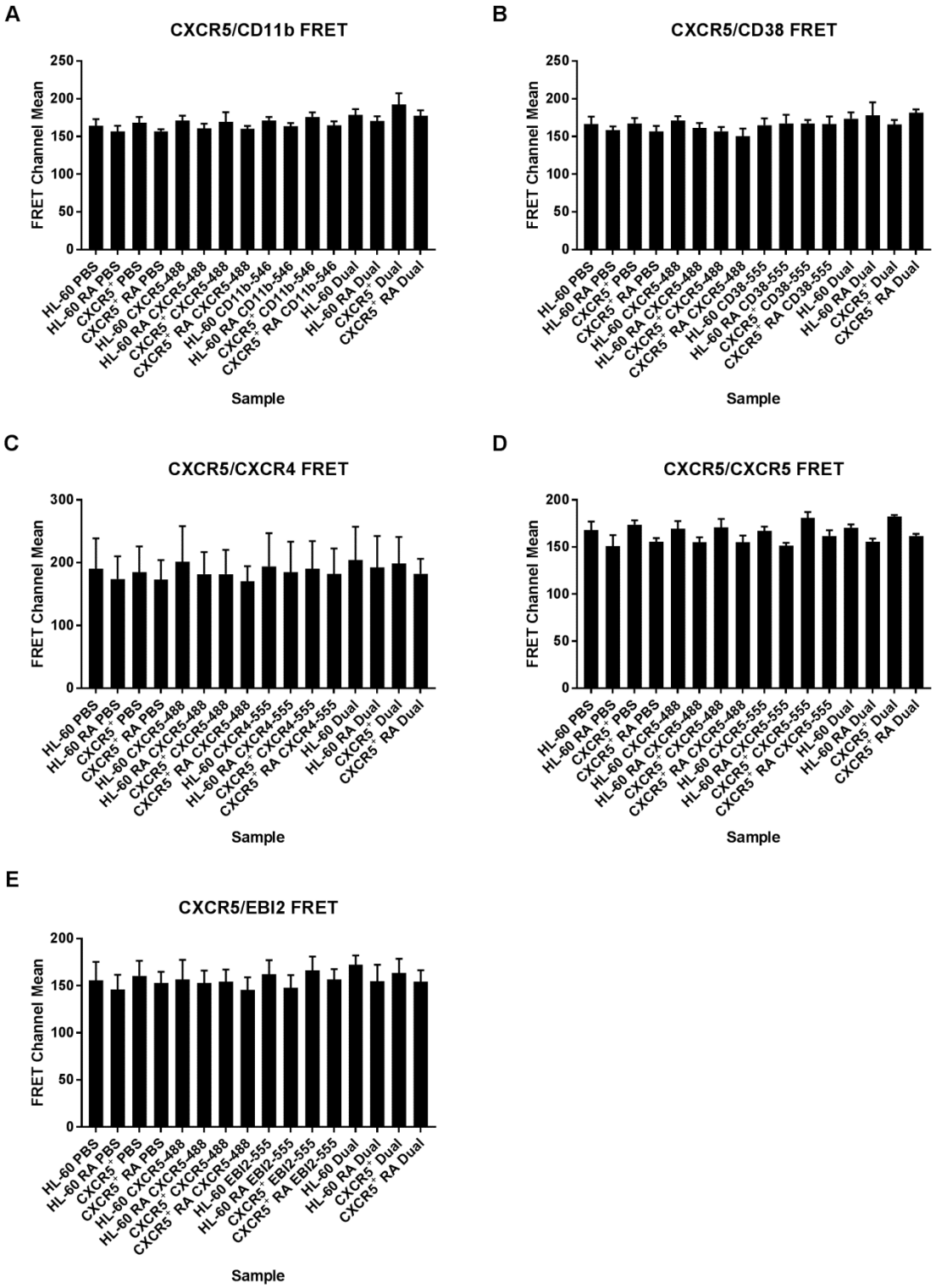

A
CXCR5/CD11b FRET
FRET Channel Mean
250
200
150
100
50
0
HL-60 PBS
HL-60 RA PBS
CXCR5+ PBS
CXCR5- RA PBS
HL-60 CXCR5-488
HL-60 RA CXCR5-488
CXCR5+ CXCR5-488
CXCR5- RA CXCR5-488
HL-60 CD11b-546
HL-60 RA CD11b-546
CXCR5+ CD11b-546
CXCR5- RA CD11b-546
HL-60 Dual
HL-60 RA Dual
CXCR5+ Dual
CXCR5- RA Dual
Sample
B
CXCR5/CD38 FRET
FRET Channel Mean
250
200
150
100
50
0
HL-60 PBS
HL-60 RA PBS
CXCR5+ PBS
CXCR5- RA PBS
HL-60 CXCR5-488
HL-60 RA CXCR5-488
CXCR5+ CXCR5-488
CXCR5- RA CXCR5-488
HL-60 CD38-555
HL-60 RA CD38-555
CXCR5+ CD38-555
CXCR5- RA CD38-555
HL-60 Dual
HL-60 RA Dual
CXCR5+ Dual
CXCR5- RA Dual
Sample
C
CXCR5/CXCR4 FRET
FRET Channel Mean
300
200
100
0
HL-60 PBS
HL-60 RA PBS
CXCR5+ PBS
CXCR5- RA PBS
HL-60 CXCR5-488
HL-60 RA CXCR5-488
CXCR5+ CXCR5-488
CXCR5- RA CXCR5-488
HL-60 CXCR4-555
HL-60 RA CXCR4-555
CXCR5+ CXCR4-555
CXCR5- RA CXCR4-555
HL-60 Dual
HL-60 RA Dual
CXCR5+ Dual
CXCR5- RA Dual
Sample
D
CXCR5/CXCR5 FRET
FRET Channel Mean
200
150
100
50
0
HL-60 PBS
HL-60 RA PBS
CXCR5+ PBS
CXCR5- RA PBS
HL-60 CXCR5-488
HL-60 RA CXCR5-488
CXCR5+ CXCR5-488
CXCR5- RA CXCR5-488
HL-60 CXCR5-555
HL-60 RA CXCR5-555
CXCR5+ CXCR5-555
CXCR5- RA CXCR5-555
HL-60 Dual
HL-60 RA Dual
CXCR5+ Dual
CXCR5- RA Dual
Sample
E
CXCR5/EBI2 FRET
FRET Channel Mean
200
150
100
50
0
HL-60 PBS
HL-60 RA PBS
CXCR5+ PBS
CXCR5- RA PBS
HL-60 CXCR5-488
HL-60 RA CXCR5-488
CXCR5+ CXCR5-488
CXCR5- RA CXCR5-488
HL-60 EBI2-555
HL-60 RA EBI2-555
CXCR5+ EBI2-555
CXCR5- RA EBI2-555
HL-60 Dual
HL-60 RA Dual
CXCR5+ Dual
CXCR5- RA Dual
Sample

Figure 4.3: Flow cytometry FRET between CXCR5 and several membrane proteins. Primary antibodies conjugated to Alexa Fluor 488 (donor) or Alexa Fluor 546/555 (acceptor) were used to label CXCR5 and candidate interaction partners on the cell surface. Mean fluorescence intensity (n = 3) in the FRET channel are shown for each pair in HL-60 and CXCR5^{+} cells with or without 24 h RA treatment as indicated. Samples were stained with PBS, single antibodies, or both antibodies (dual). Candidate CXCR5 interaction partners tested were CD11b (A), CD38 (B), CXCR4 (C), CXCR5 (D), and EBI2 (E).

4.4-4 No detectable FRET signal between CXCR5 and cytosolic protein candidates, c-Cbl and Lyn

We used flow cytometry-based FRET to test whether CXCR5 interacts with c-Cbl and Lyn, two cytosolic proteins important for RA-induced differentiation. CXCR5 has been found to cause MAPK signaling and c-Cbl and Lyn are known regulators of MAPK signaling pathways.[2,27,34,35] Both proteins are present in a signalsome driven by MAPK signaling dependent on CXCR5.[26,27] HL-60 and CXCR5^{+} cells with or without 24 h RA treatment were analyzed. As shown in Figure 4.4, we observed no enhancement of signal in the FRET channels above background, unstained levels. Hence there was no FRET indicated immediate association of CXCR5 with these cytosolic signaling regulators, where the typical detectable FRET distance is approximately 10 nM.[36]

Figure 4

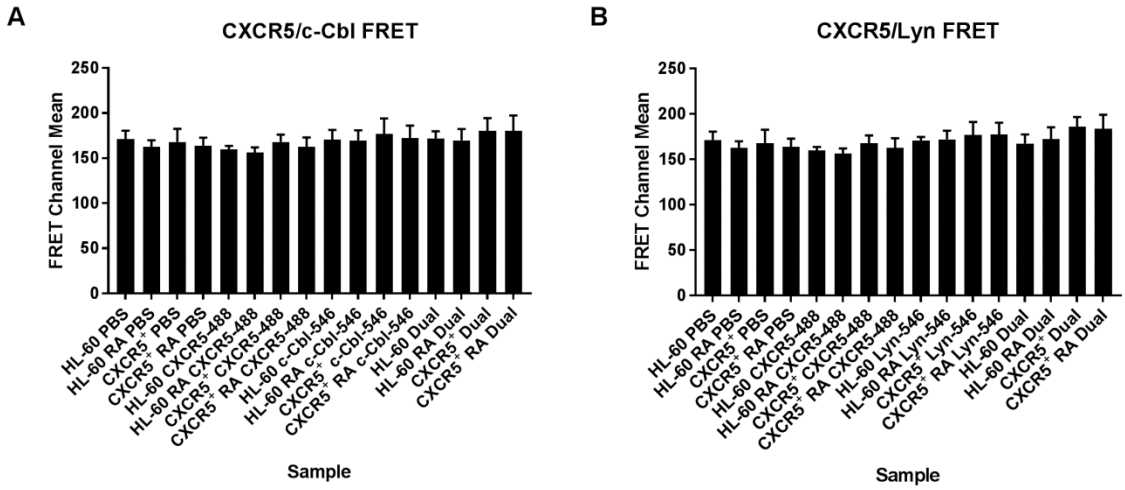


Figure 4.4: Flow cytometry FRET between CXCR5 and two cytosolic proteins. Primary antibodies conjugated to Alexa Fluor 488 (donor) or Alexa Fluor 546/555 (acceptor) were used to label CXCR5 and candidate interaction partners on the cell surface. FRET means (n = 3) are shown for each pair in HL-60 and $CXCR5^+$ cells with or without 24 h RA treatment as indicated. Samples were stained with PBS, single antibodies, or both antibodies (dual). Candidate CXCR5 interaction partners tested were c-Cbl (A) and Lyn (B).

4.4-5 Differentiation-associated signaling protein expression is not affected by CXCR5 overexpression

We sought to test whether CXCR5 overexpression might affect members of an ensemble of MAPK proteins known to play roles in RA-induced differentiation. RA-induced differentiation is driven by a signalsome that incorporates a c-Raf/MEK/ERK axis that that undergoes sustained activation and drives differentiation.[37] We treated HL-60 and $CXCR5^+$ cells for 48 h with or without 1 μM RA, collected total cell lysate, and performed western blots probing for c-Raf, MEK, ERK, and their activated phospho-proteins. We did not observe differences in expression or phosphorylation of the proteins assessed between HL-60 and $CXCR5^+$ cells; representative blots are shown in Figure 4.5. Levels of total MEK and total ERK remained constant following RA treatment, whereas levels of total c-Raf, c-Raf phosphorylated at S621, phosphorylated MEK, and phosphorylated ERK were enhanced. GAPDH was used as a loading control.

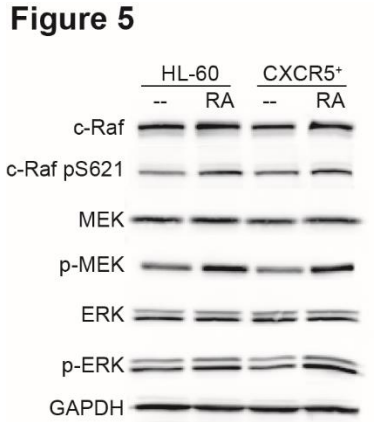


Figure 4.5: CXCR5$^+$ cells differentiate similarly to HL-60 cells upon RA treatment at several concentrations. A. HL-60 and CXCR5$^+$ cells were treated with 0.1, 0.5, or 1 µM RA over 72 h as indicated. CD11b expression was assessed by flow cytometry at 24, 48, and 72 h. Gating was set to exclude 95% of stained control cells (HL-60). n = 3. B. CD38 expression was assessed at 8, 24, and 48 h. C. Cell counts were performed using a hemocytometer and 0.2% Trypan Blue exclusion staining at 24, 48, and 72 h after treatment. D. G_1/G_0 cell cycle arrest was measured by flow cytometry using propidium iodide (PI) staining at 24, 48, and 72 h. Error bars indicate SD.

4.4-6 Overexpression of CXCR5 does not alter RA-induced differentiation

Having established that the ectopically expressed CXCR5 was functional, we characterised the effects of high levels of ectopic CXCR5 on RA-induced differentiation. We compared RA-induced differentiation in HL-60 cells to that of $CXCR5^+$ cells over a 72 h treatment period, using several myeloid lineage markers as indicators of maturation. We used several different concentrations of RA, from 0.1 to the standard 1 μM, in order to capture effects of CXCR5 overexpression that may only be apparent when using weaker RA stimulation. We assessed CD11b cell surface expression, G_1/G_0 cell cycle arrest, and cell density at 24, 48, and 72 h after treatment, while CD38 cell surface expression was evaluated at 8, 24, and 48 h after treatment since it is an early marker of differentiation.

As shown in Figure 4.6, while some of the markers, such as CD11b, exhibited a dose-response relationship with varying concentrations of RA, there were no significant differences between HL-60 and $CXCR5^+$ cells. CD38 levels after 8 h of treatment were higher in the $CXCR5^+$ cells, but these differences are not statistically significant. Interestingly there was a consistent slight enhancement, albeit not statistically significant, of cell density/growth in RA-treated cells associated with ectopic CXCR5 expression. This is consistent with the other data indicating functionality of the ectopic CXCR5, but the molecular mechanism here is enigmatic.

Figure 6

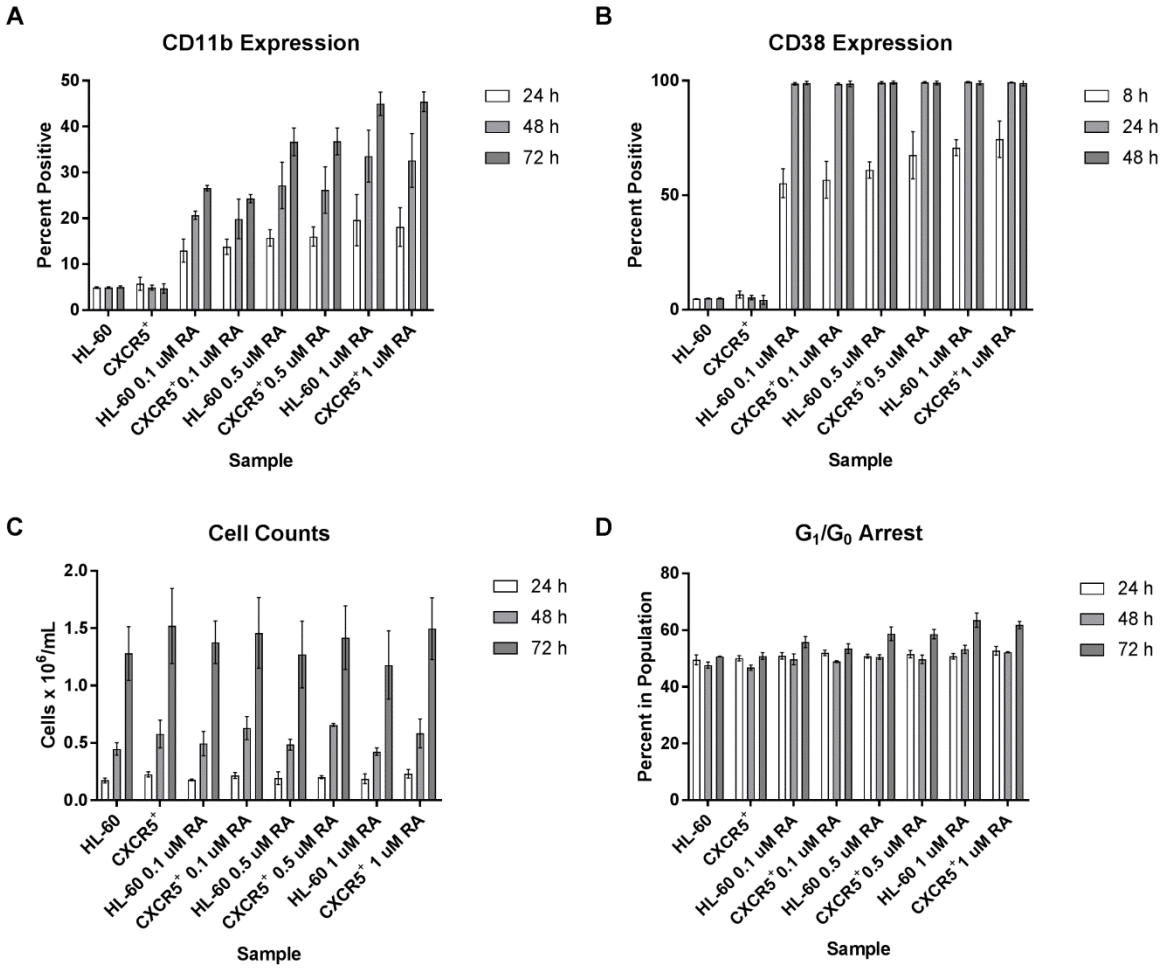


Figure 4.6: Levels and phosphorylation status of several MAPK proteins are similar in CXCR5$^+$ cells and HL-60 cells with and without RA treatment. CXCR5+ and HL-60 cells were treated with 1 µM RA for 48 h as indicated. Lysate was collected and western blots were performed, probing for c-Raf, c-Raf pS621, MEK, pMEK, ERK, and pERK. GAPDH was used as a loading control. 25 µg of lysate was loaded per lane; representative images cropped to show the band of interest are shown. n = 3 biological replicates.

4.5 Discussion

While CXCR5 is known to be capable of generating a MAPK signal, the mechanistic details are not known. Such an understanding is of import to the biology of cell proliferation and differentiation. Previous reports have implicated the protein in RA-induced differentiation of HL-60 cells through its ability to activate MAPK signaling.[2,17] A durable MAPK signaling is known to be necessary to drive cell cycle arrest and differentiation.[37–40] Knocking out CXCR5 cripples differentiation, and ectopic expression can enhance it.[2,17,18] Here, we sought to explore binding partners of CXCR5 that may help to explain its role in RA-induced differentiation. In particular, given that dimerization is one of the classical means of starting receptor signaling, we explored the conjecture that RA-induced cell surface molecules might collaborate by dimerization with CXCR5 to generating the sustained MAPK signaling involved in cell differentiation driven by RA.

We first created stable HL-60 transfectants overexpressing CXCR5, $CXCR5^+$ cells. As shown in Figure 4.1, $CXCR5^+$ cells express CXCR5 at levels well above that of RA-treated HL-60 cells as well as of a positive control, the Raji cell line. We then established that the overexpressed CXCR5 was functional by showing it enhanced migration toward CXCL13 – a known attribute of the receptor. $CXCR5^+$ cells demonstrated strong chemotaxis toward CXCL13, especially at the 0.5 µg/mL concentration, as shown in Figure 4.2. HL-60 cells, which do not express CXCR5, did not migrate toward the ligand. When treated with RA to induce modest expression of CXCR5, HL-60 cells exhibited a milder degree of migration with a dose-response curve similar to that of the $CXCR5^+$ cells. There was a background level of migration in RA-treated HL-60 cells, however; this may be due to expression of a number of other cell surface receptors that could drive migration toward

other factors in the media. Interestingly, the propensity of $CXCR5^+$ and RA-treated HL-60 cells to migrate toward CXCL13 diminished at the highest dose of CXCL13 tested, suggesting that activity in response to CXCL13 may follow a bell-shaped curve. The results suggest that the CXCR5 receptor may function – as do other receptors dependent for example on partners and relative stoichiometry therewith – in a dose dependent manner.

We next probed a set of candidate interaction partners in an attempt to determine if a classical receptor dimerization process was connecting CXCR5 to downstream MAPK signaling. We performed several flow cytometry-based FRET experiments, shown in the Figures 4.3 and 4.4, using a previously established experimental protocol for detecting protein dimerization.[29] This method, based on directly conjugating Alexa Fluor dyes to primary antibodies against targets of interest, detected CD38 dimerization as described in a previous report.[22] However, we did not detect interactions between CXCR5 and any of our candidates, a combination of surface markers and downstream proteins implicated in RA-induced differentiation or shown to interact with CXCR5 in other cellular contexts.[20,21,23–27] The candidate cell surface proteins were either implicated in other contexts or RA-induced moieties that might rationalize prolonged signal duration. The putative downstream signaling candidates tested, c-Cbl and Lyn, are regulators of the MAPK signaling pathway, the central c-Raf/MEK/ERK axes of which undergoes prolonged activation by RA to drive differentiation[34,35,37–40]. The absence of FRET argues against a direct association, but it does not preclude interactions of partners indirectly connected at a distance exceeding the Förster radius.

Our newly generated cell line did not behave as previous ectopic CXCR5 expressing lines had; we saw no phenotypic enhancement of RA-induced differentiation or associated signaling, as shown in Figures 4.5 and 4.6.[2,17,18] A number of factors may explain the differences in behavior of the presently generated cell line compared to previously described CXCR5-expressing lines. Although we used a vector with a design similar to those previously used, with an EF1α promoter and an internal ribosomal entry site linked to a neomycin resistance gene, we do not know how the expression levels of CXCR5 in the previous papers compare to those in the current report.[2,17,18] Based on the comparisons to the Raji cell line and RA-treated HL-60 cells, the levels of CXCR5 expressed in the CXCR5$^+$ line are very high. It is possible that the dose-response for CXCR5 receptor signaling activity and consequential cellular effects may follow a bell-shaped curve, such that low and high levels of the receptor in the presence of a given amount of ligand have little effect but moderate levels may enhance its activity and, in turn, RA-induced differentiation. This shape of dose-response curve, while it is not indicative, can be a feature of ligand-induced receptor dimerization.[41] An optimum dose for eliciting a given effect may be a telltale of complex multi-molecular signaling complexes.[42] Differential stoichiometric effects of CXCR5 with different signaling partners could contribute to potentially complex dose-dependent effects. Additionally, it is possible that serum levels of the CXCR5 ligand, CXCL13, were not identical in the present and previous reports.[2,17,18] CXCL13 levels in fetal bovine serum are not routinely assessed by manufacturers, and bovine and human CXCL13 share over 50% sequence identity, so it is plausible that bovine CXCL13 may be able to act as a ligand for human CXCR5.[43]

Here, we report that FRET revealed no interaction between CXCR5 and a small ensemble of candidates that may have helped explain its role in RA-induced differentiation in HL-60 cells. This includes the surface receptors CD11b and CD38, the cytosolic Src family kinase Lyn, the scaffolding protein and E3 ubiquitin ligase c-Cbl, and several partners previously reported in different cellular contexts: CXCR4, EBI2, and CXCR5 itself.[20,21] Our results suggest that CXCR5 may not directly interact with these proteins in the HL-60 context. Given its role in RA-induced differentiation of an AML cell line and that its ligand, CXCL13, is overexpressed in the context of a variety of infections, including those of *Helicobacter pylori*, *Treponema pallidum*, and *Borrelia* spp., greater understanding of CXCR5 signaling is of significance in that it may ultimately yield actionable targets for therapeutic use.[13,15,44–46] Further studies that extend the present report to elucidate the mechanism by which CXCR5 is able to generate a downstream MAPK signal are thus called for.

REFERENCES

1. Dobner, T., Wolf, I., Emrich, T. & Lipp, M. Differentiation-specific expression of a novel G protein-coupled receptor from Burkitt's lymphoma. *Eur. J. Immunol.* **22,** 2795–2799 (1992).
2. Battle, T. E., Levine, R. A. & Yen, A. Retinoic acid-induced blr1 expression promotes ERK2 activation and cell differentiation in HL-60 cells. *Exp. Cell Res.* **254,** 287–298 (2000).
3. Förster, R. *et al.* A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* **87,** 1037–1047 (1996).
4. Suto, H., Katakai, T., Sugai, M., Kinashi, T. & Shimizu, A. CXCL13 production by an established lymph node stromal cell line via lymphotoxin-beta receptor engagement involves the cooperation of multiple signaling pathways. *Int. Immunol.* **21,** 467–476 (2009).
5. Bénézech, C. *et al.* Inflammation-induced formation of fat-associated lymphoid clusters. *Nat. Immunol.* **16,** 819–828 (2015).
6. Legler, D. F. *et al.* B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J. Exp. Med.* **187,** 655–660 (1998).
7. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* **192,** 1545–1552 (2000).
8. Schaerli, P. *et al.* CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J. Exp. Med.* **192,** 1553–1562 (2000).
9. Moser, B. CXCR5, the defining marker for follicular B helper T (T_{FH}) cells. *Front. Immunol.* **6,** 296 (2015).
10. Kim, C. H. *et al.* Unique gene expression program of human germinal center T helper cells. *Blood* **104,** 1952–1960 (2004).
11. Ansel, K. M. *et al.* A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* **406,** 309–314 (2000).
12. Patel, L., Charlton, S. J., Chambers, J. K. & Macphee, C. H. Expression and functional analysis of chemokine receptors in human peripheral blood leukocyte populations. *Cytokine* **14,** 27–36 (2001).
13. Rupprecht, T. A. *et al.* Borrelia garinii induces CXCL13 production in human monocytes through toll-like receptor 2. *Infect. Immun.* **75,** 4351–4356 (2007).
14. King, M. *et al.* CXCL13 expression in Chlamydia trachomatis infection of the female reproductive tract. *Drugs Today (Barc).* **45 Suppl B,** 125–134 (2009).
15. Nakashima, Y. *et al.* Enhanced expression of CXCL13 in human helicobacter pylori-associated gastritis. *Dig. Dis. Sci.* **56,** 2887–2894 (2011).
16. Breitman, T. R., Selonick, S. E. & Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2936–2940 (1980).
17. Wang, J. & Yen, A. A MAPK-positive feedback mechanism for BLR1 signaling propels retinoic acid-triggered differentiation and cell cycle arrest. *J. Biol. Chem.* **283,** 4375–4386 (2008).

18. Wang, J. & Yen, A. A novel retinoic acid-responsive element regulates retinoic acid-induced BLR1 expression. *Mol. Cell. Biol.* **24,** 2423–2443 (2004).
19. Müller, G. & Lipp, M. Signal transduction by the chemokine receptor CXCR5: Structural requirements for G protein activation analyzed by chimeric CXCR1/CXCR5 molecules. *Biol. Chem.* **382,** 1387–1397 (2001).
20. El-Haibi, C. P. *et al.* Differential G protein subunit expression by prostate cancer cells and their interaction with CXCR5. *Mol. Cancer* **12,** 64 (2013).
21. Barroso, R. *et al.* EBI2 regulates CXCL13-mediated responses by heterodimerization with CXCR5. *FASEB J.* **26,** 4841–4854 (2012).
22. MacDonald, R. J. *et al.* Probing the requirement for CD38 in retinoic acid-induced HL-60 cell differentiation with a small molecule dimerizer and genetic knockout. *Sci. Rep.* **7,** 17406 (2017).
23. Hickstein, D. D., Back, A. L. & Collins, S. J. Regulation of expression of the CD11b and CD18 subunits of the neutrophil adherence receptor during human myeloid differentiation. *J. Biol. Chem.* **264,** 21812–21817 (1989).
24. Kontani, K., Nishina, H., Ohoka, Y., Takahashi, K. & Katada, T. NAD glycohydrolase specifically induced by retinoic acid in human leukemic HL-60 cells. Identification of the NAD glycohydrolase as leukocyte cell surface antigen CD38. *J. Biol. Chem.* **268,** 16895–16898 (1993).
25. Lamkin, T. J. *et al.* Retinoic acid-induced CD38 expression in HL-60 myeloblastic leukemia cells regulates cell differentiation or viability depending on expression levels. *J. Cell. Biochem.* **97,** 1328–1338 (2006).
26. Shen, M. & Yen, A. c-Cbl interacts with CD38 and promotes retinoic acid-induced differentiation and G0 arrest of human myeloblastic leukemia cells. *Cancer Res.* **68,** 8761–8769 (2008).
27. Congleton, J., MacDonald, R. & Yen, A. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. *Leukemia* **26,** 1180–1188 (2012).
28. Lee, B. P.-L. *et al.* CXCR5/CXCL13 interaction is important for double-negative regulatory T cell homing to cardiac allografts. *J. Immunol.* **176,** 5276–5283 (2006).
29. Förster, T. Intermolecular energy transference and fluorescence. *Ann. Phys.* **2,** 55–75 (1948).
30. Bunaciu, R. P. *et al.* 6-formylindolo(3,2-b)carbazole (FICZ) modulates the signalsome responsible for RA-induced differentiation of HL-60 myeloblastic leukemia cells. *PLoS One* **10,** e0135668 (2015).
31. Yen, A., Reece, S. L. & Albright, K. L. Control of cell differentiation during proliferation - II. Myeloid differentiation and cell cycle arrest of HL-60 promyelocytes preceded by nuclear structural changes. *Leuk. Res.* **9,** 51–71 (1985).
32. Kanbe, K., Shimizu, N., Soda, Y., Takagishi, K. & Hoshino, H. A CXC chemokine receptor, CXCR5/BLR1, is a novel and specific coreceptor for human immunodeficiency virus type 2. *Virology* **265,** 264–273 (1999).
33. Ebert, L. M., Schaerli, P. & Moser, B. Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues. *Mol. Immunol.* **42,** 799–809 (2005).
34. Swaminathan, G. & Tsygankov, A. Y. The Cbl family proteins: Ring leaders in regulation of cell signaling. *J. Cell. Physiol.* **209,** 21–43 (2006).

35. Wei, S. *et al.* IL-2 induces the association of IL-2Ro, lyn, and MAP kinase ERK-1 in human neutrophils. *Immunobiology* **202,** 363–382 (2000).
36. Shrestha, D., Jenei, A., Nagy, P., Vereb, G. & Szöllősi, J. Understanding FRET as a research tool for cellular studies. *Int. J. Mol. Sci.* **16,** 6718–6756 (2015).
37. Yen, A., Roberson, M. S., Varvayanis, S. & Lee, A. T. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res.* **58,** 3163–3172 (1998).
38. Traverse, S., Gomez, N., Paterson, H., Marshall, C. & Cohen, P. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem. J.* **288 Pt 2,** 351–355 (1992).
39. Yen, A., Williams, M., Platko, J. D., Der, C. & Hisaka, M. Expression of activated RAF accelerates cell differentiation and RB protein down-regulation but not hypophosphorylation. *Eur. J. Cell Biol.* **65,** 103–113 (1994).
40. Yen, A. & Varvayanis, S. Retinoic acid increases amount of phosphorylated RAF; ectopic expression of cFMS reveals that retinoic acid-induced differentiation is more strongly dependent on ERK2 signaling than induced GO arrest is. *In Vitro Cell. Dev. Biol. Anim.* **36,** 249–255 (2000).
41. Atanasova, M. & Whitty, A. Understanding cytokine and growth factor receptor activation mechanisms. *Crit. Rev. Biochem. Mol. Biol.* **47,** 502–530 (2012).
42. Onfroy, L. *et al.* G protein stoichiometry dictates biased agonism through distinct receptor-G protein partitioning. *Sci. Rep.* **7,** 7885 (2017).
43. Bateman, A. *et al.* UniProt: The universal protein knowledgebase. *Nucleic Acids Res.* **45,** D158–D169 (2017).
44. Marra, C. M., Tantalo, L. C., Sahi, S. K., Maxwell, C. L. & Lukehart, S. A. CXCL13 as a cerebrospinal fluid marker for neurosyphilis in HIV-infected patients with syphilis. *Sex. Transm. Dis.* **37,** 283–287 (2010).
45. Zeng, Y. L. *et al.* CXCL13 chemokine as a promising biomarker to diagnose neurosyphilis in HIV-negative patients. *Springerplus* **5,** 743 (2016).
46. Schmidt, C. *et al.* A prospective study on the role of CXCL13 in Lyme neuroborreliosis. *Neurology* **76,** 1051–1058 (2011).

Chapter 5 Foreword

This chapter represents a continuation of work on Src family kinase inhibitors initiated by Dr. Johanna Congleton and continued most immediately thereafter by Robert MacDonald. Whereas previous studies focused on PP2 and, to a lesser extent, the clinically relevant drug, dasatinib, based on literature reviews, bosutinib appeared to be another target worthy of pursuit; it is also used clinically and has a lower toxicity profile than dasatinib. Initial phenotyping of bosutinib in HL-60 cells with or without RA was performed by Robert MacDonald (Figures 5.S1 and 5.S2), who also laid out an experimental path for early characterization encompassing the experiments performed in Figures 5.1-5.4. This characterization was performed by Victoria Ip, David Tran, and Dr. Rodica Bunaciu, and western blots for several additional proteins, shown in Figure 5.5, were planned and performed by Victoria Ip, Dr. Rodica Bunaciu, and David Dai. An early version of the manuscript was written by Victoria Ip and Dr. Rodica Bunaciu and a radical re-write was done by Robert MacDonald and Dr. Rodica Bunaciu, with edits provided by all authors. The manuscript was accepted and published online but is not yet in print; the current citation is below:

MacDonald, R. J.*, Bunaciu, R. P.*, Ip, V.*, Dai, D., Tran, D., Varner, J. D. & Yen, A. Src family kinase inhibitor bosutinib enhances retinoic acid-induced differentiation of HL-60 leukemia cells. *Leuk. Lymphoma* 1-11 (2018).

*****These authors contributed equally to this work.

Chapter 5: Src family kinase inhibitor bosutinib enhances retinoic acid-induced differentiation of HL-60 leukemia cells

5.1 Abstract

The acute promyelocytic leukemia (APL) has been treated with all-trans retinoic acid (RA) for decades. While RA has largely been ineffective in non-APL AML subtypes, co-treatments combining RA and other agents are currently in clinical trials. Using the RA-responsive non-APL AML cell line HL-60, we tested the efficacy of the Src family kinase (SFK) inhibitor bosutinib on RA-induced differentiation. HL-60 has been recently shown to bear fidelity to a subtype of AML that respond to RA. We found that co-treatment with RA and bosutinib enhanced differentiation evidenced by increased CD11b expression, G_1/G_0 cell cycle arrest, and respiratory burst. Expression of the SFK members Fgr and Lyn was enhanced, while SFK activation was inhibited. Phosphorylation of several sites of c-Raf was increased and expression of AhR and p85 PI3K was enhanced. Expression of c-Cbl and mTOR was decreased. Our study suggests that SFK inhibition enhances RA-induced differentiation and may have therapeutic value in non-APL AML.

5.2 Introduction

Acute myeloid leukemia (AML), the most common form of acute leukemia in adults, is characterized by defective differentiation and excessive accumulation of proliferatively active progenitor cells in bone marrow and blood.[1,2] The acute promyelocytic leukemia (APL) subtype of AML is characterized by a t(15,17) cytogenetic marker resulting in the PML-RARα fusion protein seminal to a block of leukocyte differentiation at the promyelocyte stage and accounts for approximately 10–15% of all AML cases.[3,4] Once considered one of the most lethal forms of acute leukemia, the advent of all trans-retinoic

acid (RA) and arsenic trioxide therapy has revolutionized treatment of APL.[4] Now, APL is characterized by complete remission rates of 90% and cure rates of around 80%.[5]

While RA is effective in treating APL, it is not effective in treating other subtypes of AML. Moreover, many APL patients who initially respond to RA treatment relapse; subsequent RA treatment is ineffective due to RA-resistance.[6,7] It is therefore of great interest to combine RA with other agents, such as other differentiation-inducing compounds or kinase inhibitors, in order to promote RA-induced differentiation of APL as well as non-APL AML.[8,9]

Src family kinases (SFKs) are a group of enzymes that are important in leukemia cell proliferation, survival, adhesion, and differentiation.[2] SFKs have been shown to positively regulate MAPK signaling, cell proliferation, and contribute to cell transformation.[10]

SFKs are overexpressed in many cancer types and are typically associated with acute and chronic myeloid malignancies and metastasis.[11] Lyn has been found to be the primary active SFK expressed in AML cells.[12,13] However, in the RA-responsive non-APL AML cell line, patient derived HL-60 cells, expression of both Lyn and Fgr, the only SFKs we detected in these cells, are upregulated following RA treatment leading to differentiation.[14–16] SFK inhibition has been effective in slowing leukemic cell growth.[17] It is therefore of interest to determine the impact of SFK expression and activity on RA-induced differentiation therapy.

SFK inhibitors PP2 and dasatinib have been reported to enhance RA-induced differentiation.[13,14,18,19] However, some reports demonstrate that SFKs themselves positively regulate RA-induced differentiation.[15,16]

It is ergo unclear what role SFKs themselves have in regulating RA-induced differentiation. One focus of interest is the impact of SFK inhibitors on the MAPK pathway, where different signaling/phosphorylation signatures on the same targeted signaling molecules can cause different outcomes. Defining these signatures is important to understanding the differential signaling attributes that might be exploited for therapeutic intervention. RA has been found to elicit MAPK pathway activation necessary for HL-60 cell differentiation and growth arrest.[20] PP2 and dasatinib co-treatments with RA were found to affect the Raf/MEK/ERK axis by upregulating c-Raf pS259 while not impacting MEK or ERK expression or phosphorylation.[14] The results are somewhat counterintuitive but suggest signaling events that are of importance to inducing differentiation of the leukemic cells. They motivate interest in signaling that drives differentiation, and, in particular, they motivate targeting SFKs to probe for signaling attributes driving differentiation.

Bosutinib is a second-generation SFK inhibitor that has been used clinically to treat phases of intolerant or resistant Philadelphia chromosome (t(19,21)/Bcr-Abl) – positive chronic myeloid leukemias (CML) that do not harbor the T315I or V299LABL kinase domain mutations.[21] Bosutinib treatment has been studied extensively in CML, but its effects on AML have not been well characterized. Additionally, although the effects of SFK inhibition on APL and AML have been widely reported using PP2, the drug is for research use only; dasatinib has also been studied but has a worse toxicity profile than that of bosutinib.[22] Studies to characterize the effects of bosutinib are therefore warranted; bosutinib's clinical usage to treat CML and benign toxicity profile make it an attractive candidate for combination treatment of AML.[21,23] We recently reported that certain AML

primary cells responded favorably following co-treatment with RA and bosutinib and that the HL-60 cell line may represent an RA-responsive non-APL AML subtype.[9] Significantly, bosutinib is already in clinical use for CML and this potential off-label application to differentiation therapy could accelerate its deployment in AML.

In the present study, we examined the effects of RA/bosutinib co-treatments on HL-60 cells to determine the effects of bosutinib on HL-60 differentiation and the MAPK and mTOR pathways. We found that RA/bosutinib enhanced RA-induced expression of the differentiation marker CD11b at 48 and 72 h and G_1/G_0 arrest at 48 h. Induced oxidative metabolism and associated marker $p47^{phox}$, a component of the NADPH oxidative metabolism machine, were also enhanced with RA/ bosutinib compared to RA. With RA/bosutinib treatment, SFK members Fgr and Lyn saw an increase in expression, while pan-SFK activation decreased. bosutinib also enhanced RA-induced c-Raf phosphorylation at S259, S621, and the C-terminal domain. bosutinib decreased expression of c-Cbl and mTOR compared to RA alone. bosutinib enhanced RA-induced expression of AhR and p85 PI3K. Hence bosutinib affected several known key signaling molecule regulators of RA-induced leukemic cell differentiation. Combined RA/ bosutinib therapy may thus be useful in differentiation therapy for AML.

5.3 Materials and methods

5.3-1 Cell culture and treatments

Reagents, unless specified otherwise, were purchased from commercial suppliers in the highest purity available. HL-60 human myeloblastic leukemia cells derived from the original patient isolates, a generous gift of Dr. Robert Gallagher, were maintained in this laboratory in RPMI 1640 supplemented with 5% heat inactivated fetal bovine serum (GE

Healthcare, Chicago, IL) and 1x antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA) in a 5% CO_2 humidified atmosphere at 37°C. The cells used were certified as mycoplasma free HL-60 by Bio-Synthesis, Lewisville, TX, in August 2017. Viability was monitored by 0.2% Trypan Blue (Invitrogen, Carlsbad, CA) exclusion and routinely exceeded 95%. Experimental cultures were initiated at a density of 0.1×106 cells/mL.

There were three treatment regimens studied: (1) untreated, (2) RA, and (3) RA/bosutinib. All-trans retinoic acid (RA) (Sigma, St. Louis, MO) was added from a 5 mM stock solution in 100% ethanol to a final concentration of 1 µM in culture. Bosutinib (Sigma, St. Louis, MO) was used from a stock of 5 mM in DMSO (Sigma) to make the final concentrations in culture indicated.

Flow cytometric phenotypic analysis

5.3-2 Flow cytometric phenotypic analysis

Immunostaining for CD11b and CD38 was performed as previously described and analyzed using a Becton Dickinson LSR II flow cytometer (Franklin Lakes, NJ). Gating was set to exclude 95% of the untreated wild-type HL-60 samples. Propidium iodide (PI) cell cycle analysis was performed as previously described.[24,25]

5.3-3 Respiratory burst quantification

Respiratory burst quantification was performed as previously described and analyzed using a Becton Dickinson LSR II flow cytometer.[25] Gating was set to exclude 95% of the DMSO-treated samples. The shift in fluorescence intensity in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) was used to determine the percent cells with the capability to generate inducible oxidative metabolites.

5.3-4 Antibodies

CD38 and CD11b for flow cytometry were from Becton Dickinson (Franklin Lakes, NJ). Lyn, Fgr, pY416-SFK, AhR, $p47^{phox}$, mTOR, c-Raf pS259, c-Raf pS621, c-Raf pS289/296/301(c-Raf pC-terminal domain), GAPDH, horseradish peroxidase anti-mouse and anti-rabbit antibodies were from Cell Signaling (Danvers, MA, USA). Total c-Raf was from Becton Dickinson. c-Cbl (C-15) and AhR (H211) were from Santa Cruz Biotechnology (Santa Cruz, CA).

5.3-5 Western blot analysis

Cells were pelleted, washed twice with PBS, and lysed with ice cold mammalian protein extraction reagent (Pierce, Rockford, IL) with protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Samples were incubated overnight at −80 °C and debris was pelleted. Protein concentration was determined using the Pierce BCA Protein Assay according to the manufacturer's protocol. Lysate was subjected to standard SDS-PAGE, using 25 µg of lysate per lane under denaturing conditions. Membranes were blocked with 5% dry nonfat milk in phosphate-buffered saline (PBS) and probed with antibodies described above. Enhanced chemiluminescence reagent (GE Healthcare, Pittsburg, PA) was used for detection. Films were scanned and bands of interest were quantified using NIH ImageJ (NIH, Bethesda, MD).

5.3-6 Statistical analysis

Statistics were analyzed using Microsoft Excel. Means of treatment groups of interest were compared using two-tailed paired-sample t-tests. The data represent the means of three repeats ± standard error of the mean (SEM). A p-value of < 0.05 was considered significant.

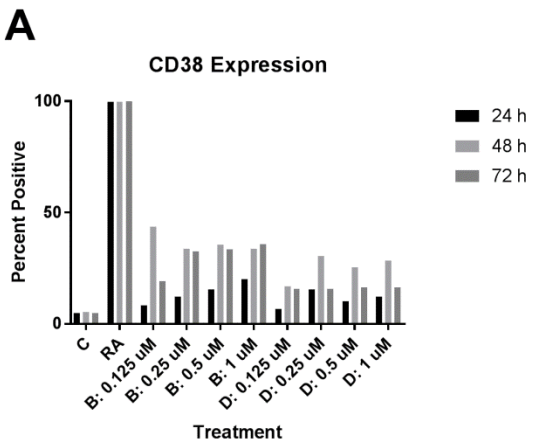
5.4 Results

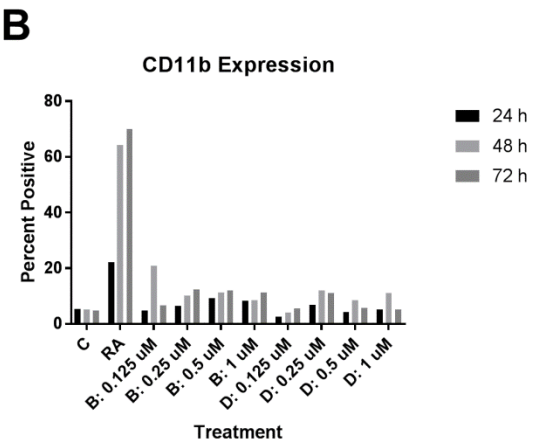
5.4-1 RA/bosutinib enhances CD11b expression and G_1/G_0 arrest

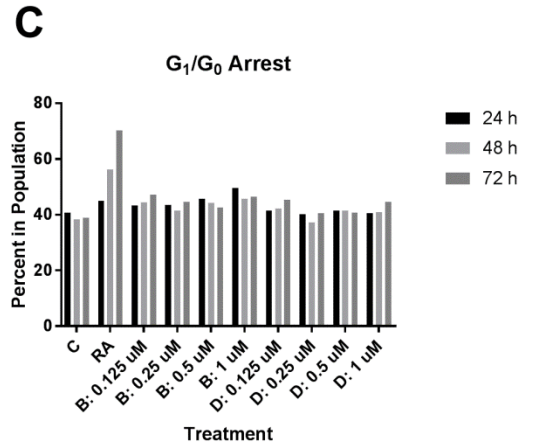
We first determined the effect of bosutinib on RA-induced differentiation in HL-60 cells by comparing differentiation markers of cells treated with RA alone or in combination with bosutinib (RA/B) over a 72 h treatment period: we assessed CD38 and CD11b expression, G_1/G_0 cell cycle arrest, and population growth. We chose to use 0.25 μM bosutinib based on an initial dose-response experiment, included in the supplementary information (Figures 5.S1 and 5.S2). It is the lowest dose that yields significant increases compared to control; i.e., the lowest observable effect level (LOEL), A previous study also found that this dosage of bosutinib inhibited cancer cell migration and invasion.[26] The dose chosen elicited no detectable toxicity or significant phenotypic shift by itself.

We measured expression of two cell surface markers, CD38 and CD11b, using flow cytometry. CD38 expression was nearly 100% in both RA and RA/bosutinib treated cells at 24, 48, and 72 hours (Figure 5.1A). CD38 mean expression per cell also yielded no difference between RA and RA/bosutinib at the three time points (Figure 5.1B). We measured CD11b, a later differentiation marker of RA-induced differentiation, at 48 h and 72 h. RA/bosutinib significantly increases CD11b expression at 48 h and 72 h (Figure 5.1C). Mean expression per cell of CD11b also shows a significant increase with RA/bosutinib treatment at 48 h (Figure 5.1D).

At 48 h but not 72 h, RA/bosutinib showed significant enhancement of G_1/G_0 arrest compared to RA alone (Figure 5.1E). Addition of bosutinib to the cells did not result in any apparent toxicity compared to RA, as both RA/bosutinib and RA treatments yield similar cell growth patterns over the 72 h treatment period (Figure 5.1F). Both RA and RA/bosutinib induce growth retardation compared to untreated cells (Figure 5.1F).


A
CD38 Expression
Percent Positive
100
50
0
24 h
48 h
72 h
C
RA
B: 0.125 uM
B: 0.25 uM
B: 0.5 uM
B: 1 uM
D: 0.125 uM
D: 0.25 uM
D: 0.5 uM
D: 1 uM
Treatment


B
CD11b Expression
Percent Positive
80
60
40
20
0
24 h
48 h
72 h
C
RA
B: 0.125 uM
B: 0.25 uM
B: 0.5 uM
B: 1 uM
D: 0.125 uM
D: 0.25 uM
D: 0.5 uM
D: 1 uM
Treatment


C
G1/G0 Arrest
Percent in Population
80
60
40
20
0
24 h
48 h
72 h
C
RA
B: 0.125 uM
B: 0.25 uM
B: 0.5 uM
B: 1 uM
D: 0.125 uM
D: 0.25 uM
D: 0.5 uM
D: 1 uM
Treatment

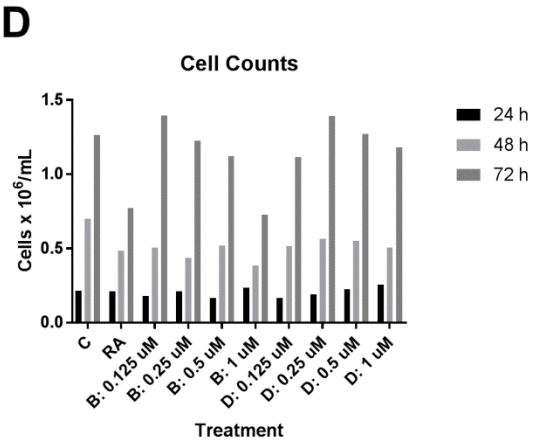
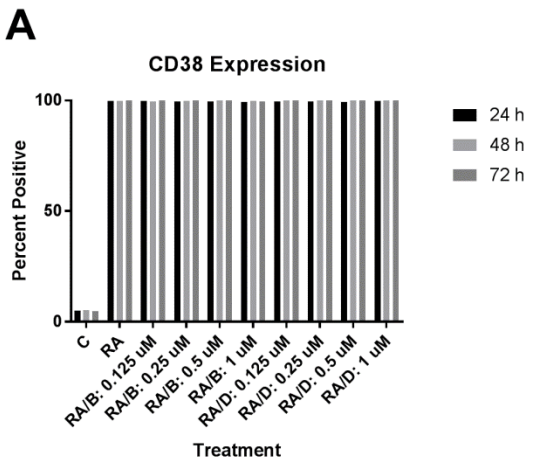
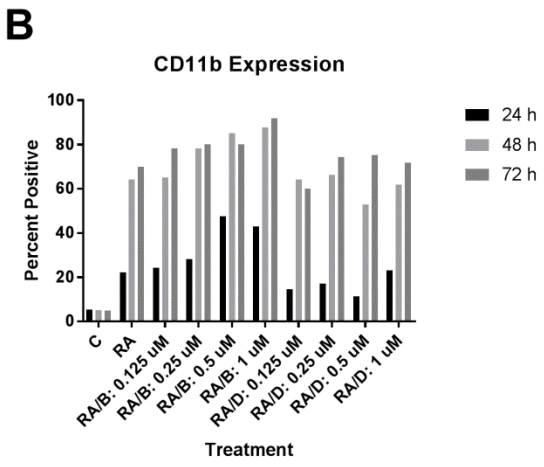
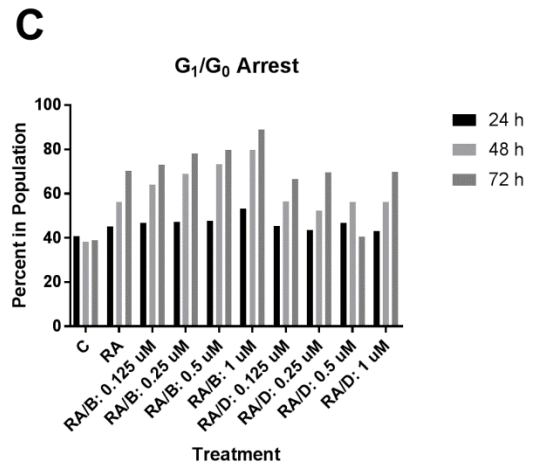

D
Cell Counts
Cells x 10^6/mL
1.5
1.0
0.5
0.0
24 h
48 h
72 h
C
RA
B: 0.125 uM
B: 0.25 uM
B: 0.5 uM
B: 1 uM
D: 0.125 uM
D: 0.25 uM
D: 0.5 uM
D: 1 uM
Treatment

Figure 5.S1: Dose-response curve of bosutinib and DMSO carrier. (A) HL-60 cells were cultured in the presence of 1 µM RA, 0.125 – 1 µM bosutinib (B) or equivalent volumes of the carrier, DMSO (D), as indicated. CD38 levels were analyzed via flow cytometry following 24, 48, and 72 h culture periods. Gating to discriminate positive cells was set to exclude 95% of untreated controls. (B) CD11b expression was assessed by flow cytometry at 24, 48, and 72 h. (C) Cell cycle distribution showing the percentage of cells in G_1/G_0 was analyzed using flow cytometry with propidium iodide staining at 24, 48, and 72 h. (D) Cell counts were taken at 24, 48, and 72 h using a hemocytometer and 0.2% Trypan Blue exclusion staining.


A
CD38 Expression
Percent Positive
100
50
0
24 h
48 h
72 h
C
RA
RA/B: 0.125 uM
RA/B: 0.25 uM
RA/B: 0.5 uM
RA/B: 1 uM
RA/D: 0.125 uM
RA/D: 0.25 uM
RA/D: 0.5 uM
RA/D: 1 uM
Treatment


B
CD11b Expression
Percent Positive
100
80
60
40
20
0
24 h
48 h
72 h
C
RA
RA/B: 0.125 uM
RA/B: 0.25 uM
RA/B: 0.5 uM
RA/B: 1 uM
RA/D: 0.125 uM
RA/D: 0.25 uM
RA/D: 0.5 uM
RA/D: 1 uM
Treatment


C
G1/G0 Arrest
Percent in Population
100
80
60
40
20
0
24 h
48 h
72 h
C
RA
RA/B: 0.125 uM
RA/B: 0.25 uM
RA/B: 0.5 uM
RA/B: 1 uM
RA/D: 0.125 uM
RA/D: 0.25 uM
RA/D: 0.5 uM
RA/D: 1 uM
Treatment

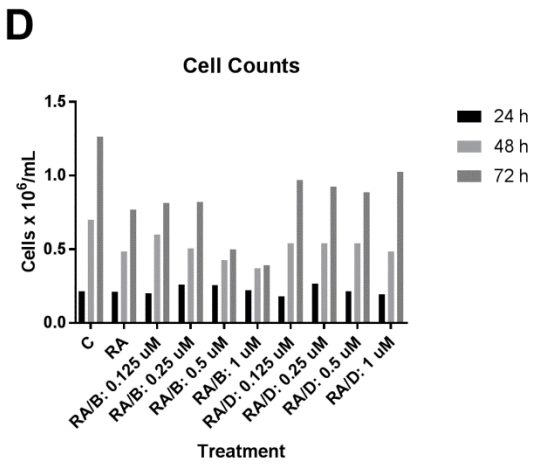

D
Cell Counts
Cells x 10^6/mL
1.5
1.0
0.5
0.0
24 h
48 h
72 h
C
RA
RA/B: 0.125 uM
RA/B: 0.25 uM
RA/B: 0.5 uM
RA/B: 1 uM
RA/D: 0.125 uM
RA/D: 0.25 uM
RA/D: 0.5 uM
RA/D: 1 uM
Treatment

Figure 5.S2: Dose-response curve of co-treatments of RA with bosutinib or DMSO carrier. (A) HL-60 cells were cultured in the presence of 1 µM RA alone or in combination with 0.125 – 1 µM bosutinib (RA/B) or equivalent volumes of the carrier, DMSO (RA/D), as indicated. CD38 levels were analyzed via flow cytometry following 24, 48, and 72 h culture periods. Gating to discriminate positive cells was set to exclude 95% of untreated controls. (B) CD11b expression was assessed by flow cytometry at 24, 48, and 72 h. (C) Cell cycle distribution showing the percentage of cells in G_1/G_0 was analyzed using flow cytometry with propidium iodide staining at 24, 48, and 72 h. (D) Cell counts were taken at 24, 48, and 72 h using a hemocytometer and 0.2% Trypan Blue exclusion staining.

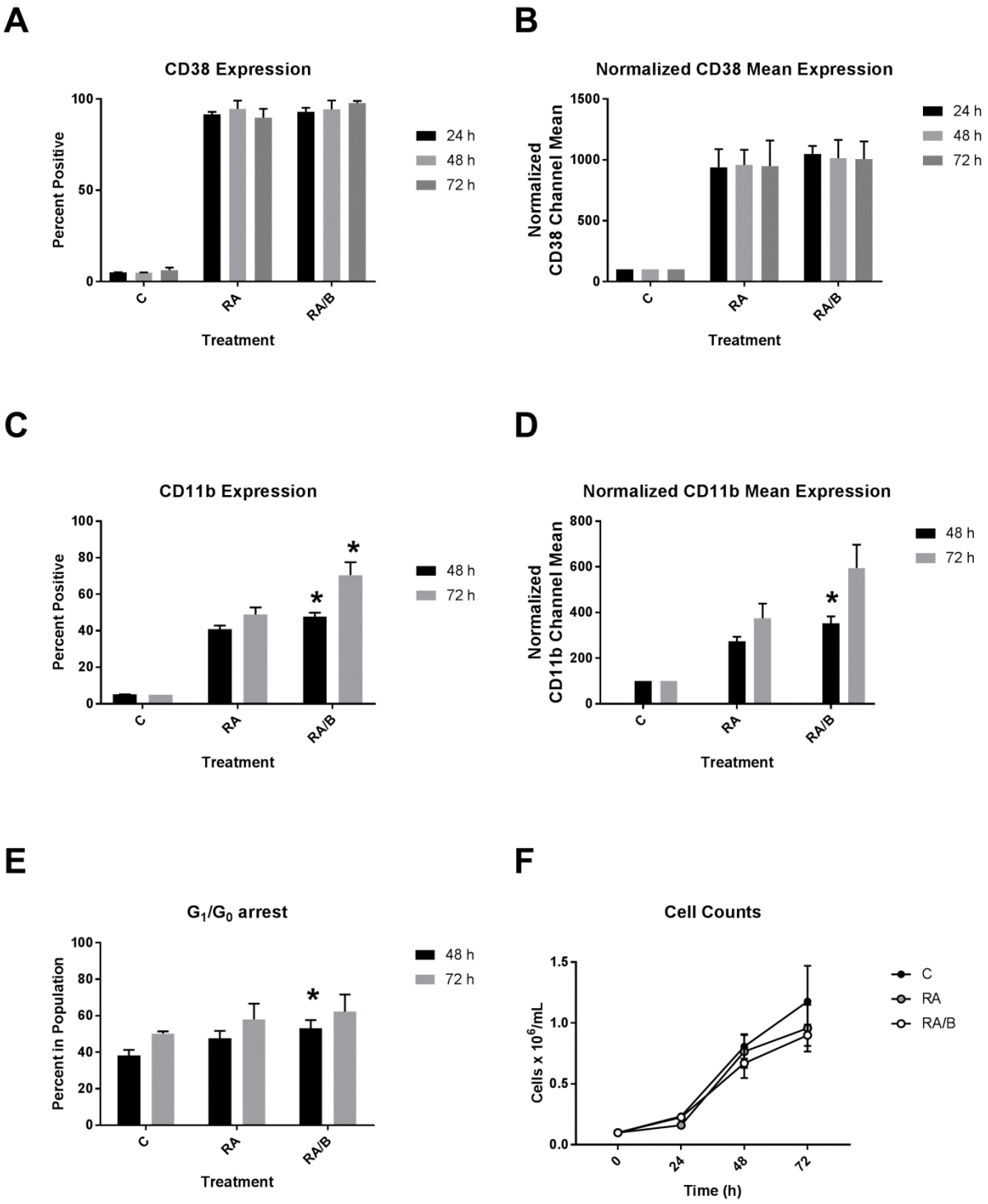
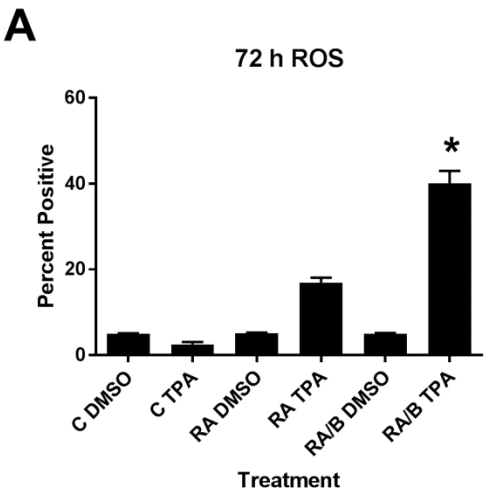

A
CD38 Expression
Percent Positive
100
50
0
C
RA
RA/B
Treatment
24 h
48 h
72 h
B
Normalized CD38 Mean Expression
Normalized CD38 Channel Mean
1500
1000
500
0
C
RA
RA/B
Treatment
24 h
48 h
72 h
C
CD11b Expression
Percent Positive
100
80
60
40
20
0
*
*
C
RA
RA/B
Treatment
48 h
72 h
D
Normalized CD11b Mean Expression
Normalized CD11b Channel Mean
800
600
400
200
0
*
C
RA
RA/B
Treatment
48 h
72 h
E
G1/G0 arrest
Percent in Population
100
80
60
40
20
0
*
C
RA
RA/B
Treatment
48 h
72 h
F
Cell Counts
Cells x 10^6/mL
1.5
1.0
0.5
0.0
0
24
48
72
Time (h)
C
RA
RA/B

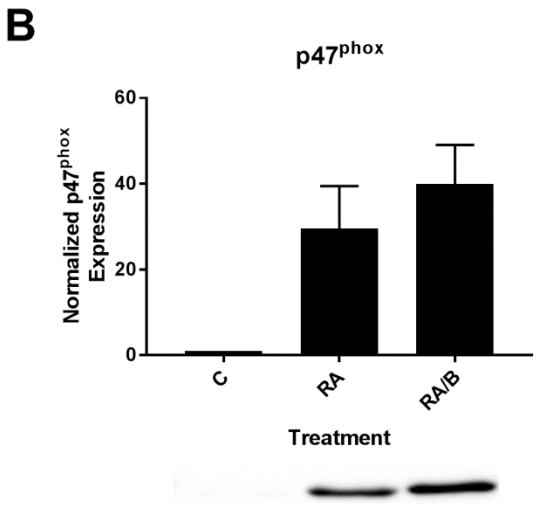
Figure 5.1: Phenotypic analysis of HL-60 cells treated with RA/bosutinib. (A) HL-60 cells were cultured in the presence of 1 µM RA or 1 µM RA and 0.25 µM bosutinib (B) as indicated. CD38 expression was assessed by flow cytometry following 24, 48, and 72 h treatment periods. Gating to discriminate positive cells was set to exclude 95% of untreated controls (n=3). Error bars indicate SEM. (B) Normalized means of CD38 expression per cell at 24 h, 48 h, or 72 h (n=3). (C) CD11b expression was assessed by flow cytometry at 48 and 72 h (n=3). *, $p < 0.05$ comparing RA-treated samples to RA/bosutinib-treated samples. Two-tailed paired-sample t-tests were used to determine significance. (D) Normalized means of CD11b expression per cell at 48 h and 72 h (n=3). *, $p < 0.05$ comparing RA-treated samples to RA/bosutinib-treated samples. (E) Cell cycle distribution showing the percentage of cells in G_1/G_0 was analyzed using flow cytometry with propidium iodide staining at 24, 48, and 72 h (n=4). *, $p < 0.05$ comparing RA-treated samples to RA/bosutinib-treated samples. (F) Cell counts were taken at 24, 48, and 72 h using a hemocytometer and 0.2% Trypan Blue exclusion staining (n=3).

5.4-2 RA/bosutinib increases RA-induced respiratory burst activity

To determine the effects of RA/bosutinib on a functional differentiation marker, respiratory burst, we measured inducible reactive oxygen species production (ROS) by flow cytometry at 72 h. RA caused an increase in ROS compared to untreated cells, and RA/bosutinib treated cells show a significant further increase in oxidative metabolism compared to RA alone (Figure 5.2A).

To corroborate the ROS assay, we also measured expression of a subunit of the NADPH oxidase complex that produces the respiratory burst, $p47^{phox}$.[27,28] After a 48 h treatment period, we collected cell lysate and performed western blots for $p47^{phox}$ (Figure 5.2B). Expression was greater in both RA and RA/bosutinib compared to untreated cells, and RA/bosutinib further enhanced $p47^{phox}$ levels compared to RA alone (Figure 5.2B). GAPDH was used as a loading control (Figure 5.2C).


A
72 h ROS
Percent Positive
60
40
20
0
*
C DMSO
C TPA
RA DMSO
RA TPA
RA/B DMSO
RA/B TPA
Treatment


B
p47phox
Normalized p47phox Expression
60
40
20
0
C
RA
RA/B
Treatment

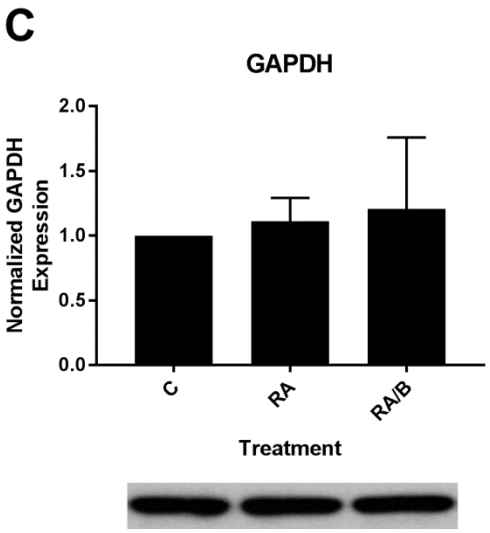

C
GAPDH
Normalized GAPDH Expression
2.0
1.5
1.0
0.5
0.0
C
RA
RA/B
Treatment

Figure 5.2: HL-60 cells treated with RA/bosutinib displayed enhanced respiratory burst and p47^{phox} expression. (A) HL-60 cells were cultured in the presence of 1 μM RA or 1 μM RA and 0.25 μM bosutinib (B) as indicated. Respiratory burst was analyzed by measuring inducible reactive oxygen species (ROS) production by flow cytometry using the DCF assay. Gates to determine percent increase of expression with treatment were set to exclude 95% of the DMSO-treated control population for each culture condition; TPA-treated samples show induced ROS (n=3). Error bars indicate SEM. *, $p < 0.05$ comparing RA-treated samples to RA/bosutinib-treated samples. Two-tailed paired-sample t-tests were used to determine significance. (B) HL-60 cells were cultured for 48 h in the presence of 1 μM RA or 1 μM RA and 0.25 μM B as indicated and whole cell lysate was collected. 25 μg of lysate per lane was run. Western blots of PAGE-resolved lysates were probed for p47^{phox} (n=3). Films were scanned and bands of interest were quantified using NIH ImageJ. Error bars indicate SEM. A representative blot, cropped to show only the band of interest, is included. (C) Western blots of GAPDH were used as loading controls following the procedure described above.

5.4-3 RA/bosutinib increases RA-induced SFK expression, but decreases SFK activation

As bosutinib is an SFK inhibitor and the SFK members Fgr and Lyn have been shown to be upregulated by RA treatment in HL-60 cells, we assessed levels and phosphorylation of these members by western blot.[3,26,28] Since Fgr and Lyn are the primary SFK members that are upregulated (i.e. Fgr, Lyn) or activated (i.e., Lyn) in response to RA treatment in AML, we did not probe for other members.[12–16] We collected lysate following a 48 h treatment period. Both RA and RA/bosutinib increased Fgr and Lyn levels compared to untreated cells, and RA/ bosutinib induced further increases of Fgr and Lyn levels compared to RA alone (Figures 5.3A and 5.3B).

We measured SFK activation using a pan-SFK antibody that detects active site (Y416) phosphorylation in all family members, including Fgr and Lyn. While treatment with RA and RA/bosutinib both caused increases in SFK phosphorylation compared to untreated cells, treatment with RA/bosutinib decreased SFK phosphorylation compared to RA alone (Figure 5.3, A-C). GAPDH was used as a loading control (Figure 5.3D).

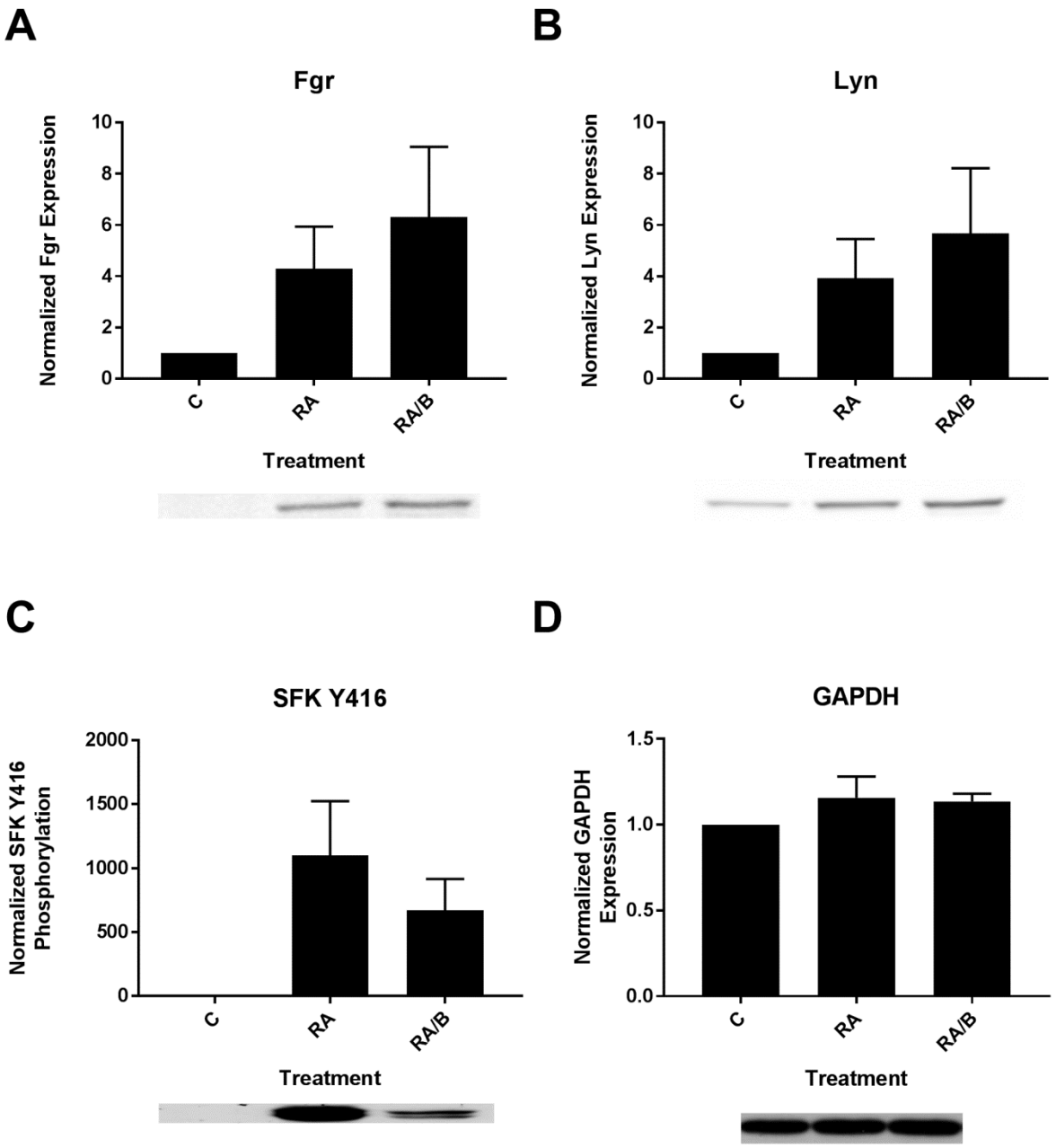

A
Fgr
Normalized Fgr Expression
10
8
6
4
2
0
C
RA
RA/B
Treatment
B
Lyn
Normalized Lyn Expression
10
8
6
4
2
0
C
RA
RA/B
Treatment
C
SFK Y416
Normalized SFK Y416 Phosphorylation
2000
1500
1000
500
0
C
RA
RA/B
Treatment
D
GAPDH
Normalized GAPDH Expression
1.5
1.0
0.5
0.0
C
RA
RA/B
Treatment

Figure 5.3: Bosutinib enhances RA-induced SFK expression and diminishes SFK phosphorylation. (A) HL-60 cells were cultured for 48 h in the presence of 1 µM RA or 1 µM RA and 0.25 µM bosutinib (B) as indicated and whole cell lysate was collected. 25 µg of lysate per lane was run. Western blots of PAGE-resolved lysates were probed for Fgr (n=3). Films were scanned and bands of interest were quantified using NIH ImageJ. Error bars indicate SEM. A representative blot, cropped to show only the band of interest, is included. (B) Western blots of Lyn following the procedure described above. (C) Western blots of phosphorylated pan-Y416 SFK following the procedure described above. (D) Western blots of GAPDH were used as loading controls following the procedure described above.

5.4-4 RA/bosutinib augments RA-induced c-Raf phosphorylation

We also examined phosphorylation of several sites of c-Raf previously shown to drive RA-induced differentiation.[29] Since SFKs can regulate MAPK signaling, we analyzed the effect of bosutinib on RA-induced c-Raf activation. We evaluated the phosphorylation status of c-Raf regulatory phosphorylations at S259, S621, and the c-Raf C-terminal domain by western blot following a 48 h treatment period. RA increases the amount of c-Raf pS259, c-Raf pS621, and c-Raf pC-terminal domain. We observed modest enhancements of levels of each of these in cells treated with RA/bosutinib compared to RA alone (Figure 5.4, A-C). Total c-Raf levels were modestly increased with both RA and RA/bosutinib treatment (Figure 5.4D). GAPDH was used as a loading control (Figure 5.4E).

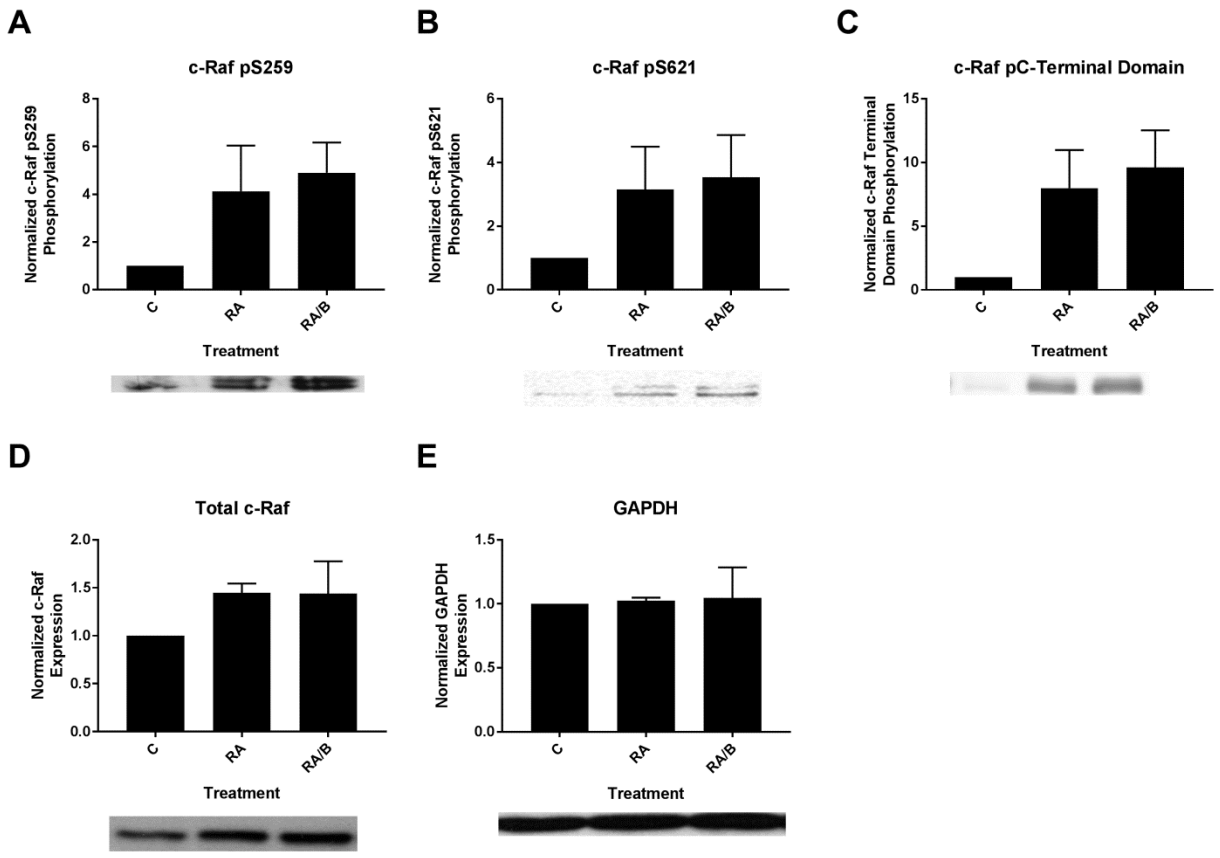


Figure 5.4: Bosutinib enhances levels of RA-induced phosphorylated c-Raf. (A) HL-60 cells were cultured for 48 h in the presence of 1 µM RA or 1 µM RA and 0.25 µM bosutinib (B) as indicated and whole cell lysate was collected. 25 µg of lysate per lane was run. Western blots of PAGE-resolved lysates were probed for c-Raf pS259 (n=3). Films were scanned and bands of interest were quantified using NIH ImageJ. Error bars indicate SEM. A representative blot, cropped to show only the band of interest, is included. (B) Western blots of c-Raf pS621 following the procedure described above. (C) Western blots of c-Raf pC-terminal domain following the procedure described above. (D) Western blots of total c-Raf following the procedure described above. (E) Western blots of GAPDH were used as loading controls following the procedure described above.

5.4-5 RA/bosutinib inhibits c-Cbl and mTOR expression and enhances AhR and p85 PI3K expression

We next assessed levels of several proteins associated with RA-induced differentiation, AhR, c-Cbl, and p85 PI3K, as well as one known to be aberrantly activated in AML, mTOR.[8,24,30,31] After a 48 h treatment period, we collected cell lysate and analyzed expression of these proteins via western blot. C-Cbl expression was upregulated in cells treated with RA and RA/ bosutinib, but RA/bosutinib inhibited c-Cbl expression compared to cells treated with RA alone (Fig. 5A). mTOR expression followed a similar pattern (Figure 5.5B). AhR and p85 PI3K levels, however, were further upregulated in RA/bosutinib -treated cells compared to RA-treated cells (Figures 5.5C and 5.5D). GAPDH was used as a loading control (Figure 5.5E).

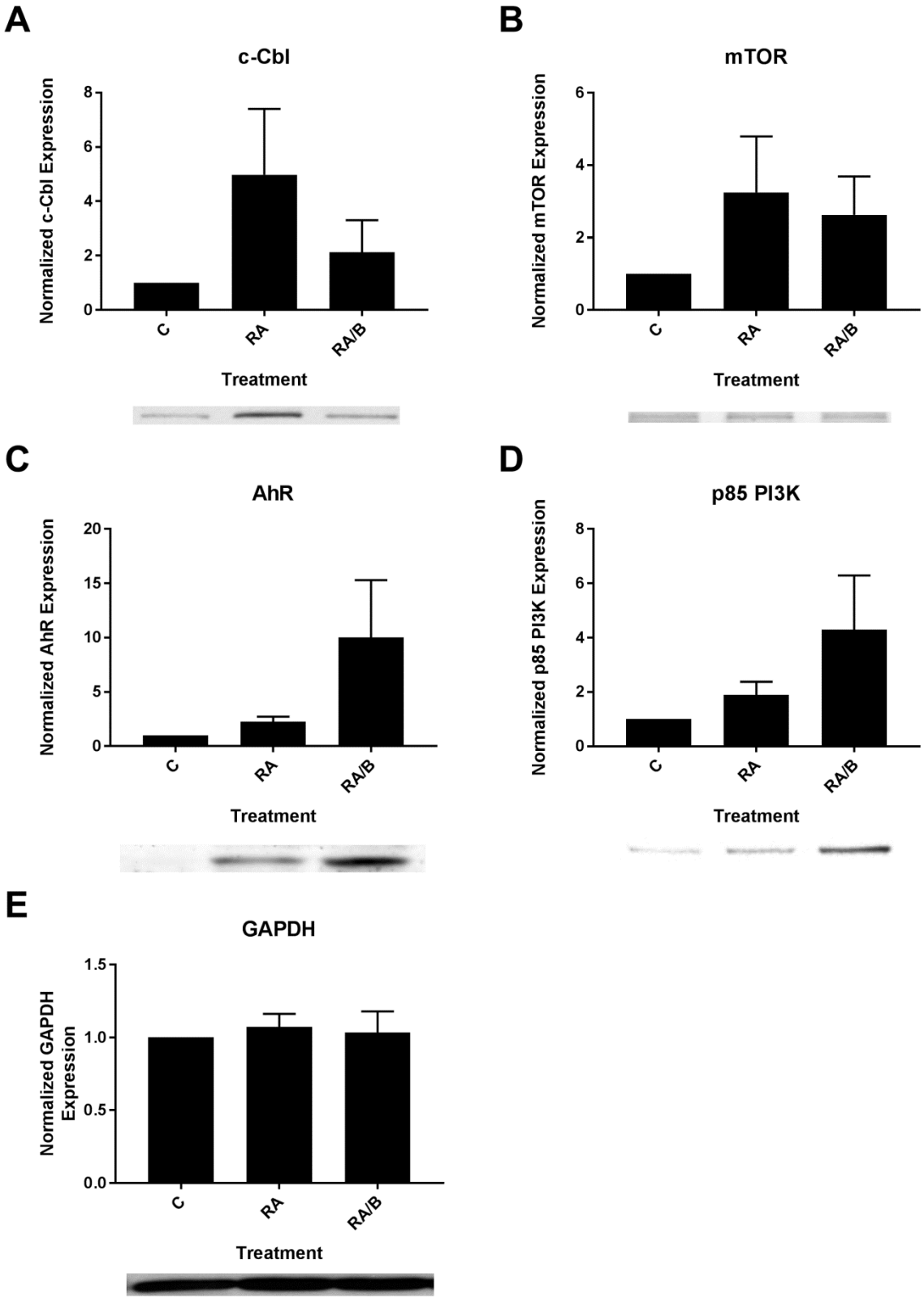

A
c-Cbl
Normalized c-Cbl Expression
0
2
4
6
8
C
RA
RA/B
Treatment
B
mTOR
Normalized mTOR Expression
0
2
4
6
C
RA
RA/B
Treatment
C
AhR
Normalized AhR Expression
0
5
10
15
20
C
RA
RA/B
Treatment
D
p85 PI3K
Normalized p85 PI3K Expression
0
2
4
6
8
C
RA
RA/B
Treatment
E
GAPDH
Normalized GAPDH Expression
0.0
0.5
1.0
1.5
C
RA
RA/B
Treatment

Figure 5.5: The effect of bosutinib on c-Cbl, mTOR, AhR, and p85 PI3K. (A) HL-60 cells were cultured for 48 h in the presence of 1 µM RA or 1 µM RA and 0.25 µM bosutinib (B) as indicated and whole cell lysate was collected. 25 µg of lysate per lane was run. Western blots of PAGE-resolved lysates were probed for c-Cbl (n=3). Films were scanned and bands of interest were quantified using NIH ImageJ. Error bars indicate SEM. A representative blot, cropped to show only the band of interest, is included. (B) Western blots of mTOR following the procedure described above. (C) Western blots of AhR following the procedure described above. (D) Western blots of p85 PI3K following the procedure described above. (E) Western blots of GAPDH were used as loading controls following the procedure described above.

5.5 Discussion

We sought to provide an initial characterization of the effects of treatment with bosutinib on RA-induced differentiation of HL-60 cells in order to explore its potential therapeutic value in non-APL AML. We recently reported that the HL-60 cell line bears fidelity to a previously undefined RA-responsive, non-APL subtype of AML.[9] Here, we observed that RA/bosutinib treatment enhanced several myeloid lineage differentiation markers compared with RA treatment alone: CD11b expression, G_1/G_0 cell cycle arrest, and respiratory burst, a functional marker of mature myeloid series cells.[28] Expression of CD38, an early marker of RA-induced differentiation, was not affected by the addition of bosutinib. Recent reports from our laboratory, however, suggest that CD38 may not be necessary for the differentiation process, although it can enhance it.[9,21,32]

Since addition of bosutinib enhanced phenotypic and functional markers of RA-induced differentiation, we explored its effects on activation of the key signaling protein c-Raf. Cells treated with RA/bosutinib displayed increased phosphorylated c-Raf levels. The particular phosphorylation sites assayed, S259, S621, and the C-terminal domain, are all associated with active c-Raf in HL-60 cells and thus drive RA-induced differentiation.[29] Our western blotting results are consistent with our phenotypic results and previous findings that RA co-treatments with PP2 or dasatinib also increased c-Raf phosphorylation, namely c-Raf pS259 and c-Raf pS621, in HL-60 cells.[14] These findings are congruous with the notion that c-Raf is a downstream target of SFK inhibitors like bosutinib.

Given that bosutinib is a SFK inhibitor, we assessed its effects on the two prominent SFK members expressed in RA-treated HL-60 cells, Fgr and Lyn, the primary active SFK

expressed in AML cells.[12–16] As anticipated, we saw that RA/ bosutinib treatment decreased levels of phosphorylated Y416, a mark of activated SFKs. We did, however, observe increased levels of Fgr and Lyn in RA/bosutinib compared to RA alone; increase in expression could perhaps be an attempt to compensate for the inhibited activity of the proteins. These results are consistent with previous findings with RA/dasatinib co-treatments.[14] Dasatinib, like bosutinib, is a second generation SFK inhibitor that is used clinically to treat CML. Bosutinib, however, may be more interesting as a potential therapeutic intervention in AML because it has a lower toxicity profile compared to dasatinib; it is used in patients with CML who have shown intolerance to dasatinib.[22]

c-Cbl interacts with CD38 to promote RA-induced differentiation and G_1/G_0 arrest of HL-60 cells.[30] As previously reported, c-Cbl expression is upregulated by RA treatment; RA/ bosutinib treatment partially inhibits RA-induced upregulation. Since c-Cbl expression has been shown to drive RA-induced differentiation, this is surprising, but it is possible that bosutinib drives RA-induced differentiation via other pathways such as c-Raf activation.

The mTOR/p70S6K/4EBP1 pathway, a driver of cellular anabolism/metabolism, is constitutively activated in AML cells, leading to increased cell proliferation and inhibition of differentiation.[33] mTOR is thought to be downstream of the pathways implicated above to regulate differentiation. Dasatinib inactivates the mTOR pathway, correlating with an improvement in cell survival in the AML-derived cell line PVTL-1.[33] Lyn is hypothesized to serve as an intermediary; Lyn stimulates mTOR expression and is inhibited by dasatinib.[13,33] Lending credence to this, silencing Lyn was found to inhibit the mTOR pathway in primary AML cells, and PP2 mimics rapamycin, a selective inhibitor of mTOR.[12]

To our knowledge, the effects of SFK inhibitors on the mTOR pathway in APL are unknown. It was hence of interest to determine if bosutinib has an inhibitory effect on mTOR in an RA-responsive AML cell line as found in other forms of AML. Both RA and RA/bosutinib induced greater mTOR expression compared to untreated cells, however, RA/bosutinib decreased mTOR expression compared to RA (Figure 5.5B). It appears that a decrease in the activated SFKs yields an inhibitory effect on the mTOR pathway in RA-treated cells. This inhibitory effect on the mTOR pathway suggests part of the process by which bosutinib enhances differentiation compared to RA alone.

We note that MAPK signaling pathway activation is promoting differentiation and cell cycle arrest in RA-treated HL-60 cells, yet it is also thought to drive mTOR and, consequentially, cellular anabolism to promote proliferation. Hence, RA appears to be redirecting these pathways to support differentiation instead of proliferation. The mechanism of this is a profound but yet unresolved question in leukemic cell differentiation.

AhR is known to drive differentiation and to be linked to phosphorylated c-Raf in an RA-activated signalsome.[25] We found that RA treatment increased AhR levels, consistent with previous studies (Figure 5.5C).[3,34] Co-treatment with bosutinib further increased AhR expression, consistent with the increase in phosphorylated c-Raf expression in RA/bosutinib treated samples (Figure 5.3, Figure 5.5C).

p85 PI3K activity is upregulated during granulocytic maturation.[35] It was therefore of interest to determine the effect of bosutinib on p85 PI3K expression; the protein exhibited an expression pattern similar to that of AhR, as bosutinib caused an increase in RA-induced upregulation of p85 PI3K expression. Interestingly, while both CD38 and c-Cbl

are known to interact with p85 PI3K, RA/bosutinib treatment did not affect the RA-induced expression of these proteins in the same fashion.[31]

Bosutinib, like other SFK inhibitors, has promising effects on enhancing RA-induced differentiation in HL-60 cells. These effects include modulation of the SFK members Fgr and Lyn and impacts on the MAPK and mTOR pathways. Further studies may elucidate the effects of RA/bosutinib co-treatments on RA-resistant APL cell lines or other myeloid leukemias. Bosutinib is already in clinical trials as a cytotoxic chemotherapeutic agent, but our results suggest it is potentially useful as an agent used in combination therapy with RA for differentiation therapy of AML.

REFERENCES

1. Hu, S. *et al.* A novel glycogen synthase kinase-3 inhibitor optimized for acute myeloid leukemia differentiation activity. *Mol. Cancer Ther.* **15,** 1485–1494 (2016).
2. Iriyama, N., Yuan, B., Hatta, Y., Takagi, N. & Takei, M. Lyn, a tyrosine kinase closely linked to the differentiation status of primary acute myeloid leukemia blasts, associates with negative regulation of all-trans retinoic acid (ATRA) and dihydroxyvitamin D3 (VD3)-induced HL-60 cells differentiation. *Cancer Cell Int.* **16,** 37 (2016).
3. Ibabao, C. N., Bunaciu, R. P., Schaefer, D. M. W. & Yen, A. The AhR agonist VAF347 augments retinoic acid-induced differentiation in leukemia cells. *FEBS Open Bio* **5,** 308–318 (2015).
4. Watts, J. M. & Tallman, M. S. Acute promyelocytic leukemia: what is the new standard of care? *Blood Rev.* **28,** 205–212 (2014).
5. Coombs, C. C., Tavakkoli, M. & Tallman, M. S. Acute promyelocytic leukemia: where did we start, where are we now, and the future. *Blood Cancer J.* **5,** e304 (2015).
6. Warrell Jr., R. P. Retinoid resistance in acute promyelocytic leukemia: new mechanisms, strategies, and implications. *Blood* **82,** 1949–1953 (1993).
7. Freemantle, S. J., Spinella, M. J. & Dmitrovsky, E. Retinoids in cancer therapy and chemoprevention: promise meets resistance. *Oncogene* **22,** 7305–7315 (2003).
8. Jensen, H. A., Bunaciu, R. P., Varner, J. D. & Yen, A. GW5074 and PP2 kinase inhibitors implicate nontraditional c-Raf and Lyn function as drivers of retinoic acid-induced maturation. *Cell. Signal.* **27,** 1666–1675 (2015).
9. Bunaciu, R. P. *et al.* Potential for subsets of wt-NPM1 primary AML blasts to respond to retinoic acid treatment. *Oncotarget* **5,** 4134–4149 (2017).
10. Kim, M. P., Park, S. I., Kopetz, S. & Gallick, G. E. Src family kinases as mediators of endothelial permeability: Effects on inflammation and metastasis. *Cell Tissue Res.* **335,** 249–259 (2009).
11. Logue, J. S. & Morrison, D. K. Complexity in the signaling network: Insights from the use of targeted inhibitors in cancer therapy. *Genes Dev.* **26,** 641–650 (2012).
12. Dos Santos, C. *et al.* A critical role for Lyn in acute myeloid leukemia. *Blood* **111,** 2269–2279 (2008).
13. Kropf, P. L., Wang, L., Zang, Y., Redner, R. L. & Johnson, D. E. Dasatinib promotes ATRA-induced differentiation of AML cells. *Leukemia* **24,** 663–665 (2010).
14. Congleton, J., MacDonald, R. & Yen, A. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. *Leukemia* **26,** 1180–1188 (2012).
15. Katagiri, K. *et al.* Lyn and Fgr protein-tyrosine kinases prevent apoptosis during retinoic acid-induced granulocytic differentiation of HL-60 cells. *J. Biol. Chem.* **271,** 11557–11562 (1996).
16. Notario, V., Gutkind, J. S., Imaizumi, M., Ketamine, S. & Robbins, K. C. Expression of the fgr protooncogene product as a function of myelomonocytic cell maturation. *J. Cell Biol.* **109,** 3129–3136 (1989).
17. Guerrouahen, B. S. *et al.* Dasatinib inhibits the growth of molecularly

heterogeneous myeloid leukemias. *Clin. Cancer Res.* **16,** 1149-1158 (2010).
18. Miranda, M. B., Redner, R. L. & Johnson, D. E. Inhibition of Src family kinases enhances retinoic acid induced gene expression and myeloid differentiation. *Mol. Cancer Ther.* **6,** 3081–3090 (2007).
19. Jensen, H. A. *et al.* The Src-family kinase inhibitor PP2 rescues inducible differentiation events in emergent retinoic acid-resistant myeloblastic leukemia cells. *PLoS One* **8,** e58621 (2013).
20. Yen, a, Roberson, M. S., Varvayanis, S. & Lee, a T. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res.* **58,** 3163–3172 (1998).
21. Abbas, R. & Hsyu, P.-H. Clinical pharmacokinetics and pharmacodynamics of bosutinib. *Clin. Pharmacokinet.* **55,** 1191-1204 (2016).
22. Kantarjian, H. M. *et al.* Bosutinib safety and management of toxicity in leukemia patients with resistance or intolerance to imatinib and other tyrosine kinase inhibitors. *Blood* **123,** 1309–1318 (2014).
23. Cortes, J. E. *et al.* Safety and efficacy of bosutinib (SKI-606) in chronic phase Philadelphia chromosome–positive chronic myeloid leukemia patients with resistance or intolerance to imatinib. *Blood* **118,** 4567-4576 (2011).
24. MacDonald, R. J. *et al.* Probing the requirement for CD38 in retinoic acid-induced HL-60 cell differentiation with a small molecule dimerizer and genetic knockout. *Sci. Rep.* **7,** 17406 (2017).
25. Bunaciu, R. P. & Yen, A. Activation of the aryl hydrocarbon receptor ahr promotes retinoic acid-induced differentiation of myeloblastic leukemia cells by restricting expression of the stem cell transcription factor Oct4. *Cancer Res.* **71,** 2371–2380 (2011).
26. Vultur, A. *et al.* SKI-606 (bosutinib), a novel Src kinase inhibitor, suppresses migration and invasion of human breast cancer cells. *Mol. Cancer Ther.* **7,** 1185–1194 (2008).
27. Wallace, A. S. *et al.* RRD-251 enhances All-trans retinoic acid (RA)-induced differentiation of HL-60 myeloblastic leukemia cells. *Oncotarget* **7,** 46401–46418 (2016).
28. Jensen, H. A. *et al.* Retinoic acid therapy resistance progresses from unilineage to bilineage in HL-60 leukemic blasts. *PLoS One* **9,** e98929 (2014).
29. Yen, A., Williams, M., Platko, J. D., Der, C. & Hisaka, M. Expression of activated RAF accelerates cell differentiation and RB protein down-regulation but not hypophosphorylation. *Eur. J. Cell Biol.* **65,** 103–113 (1994).
30. Shen, M. & Yen, A. c-Cbl interacts with CD38 and promotes retinoic acid-induced differentiation and G0 arrest of human myeloblastic leukemia cells. *Cancer Res.* **68,** 8761–8769 (2008).
31. Congleton, J., Shen, M., MacDonald, R., Malavasi, F. & Yen, A. Phosphorylation of c-Cbl and p85 PI3K driven by all-trans retinoic acid and CD38 depends on Lyn kinase activity. *Cell. Signal.* **26,** 1589–1597 (2014).
32. Lamkin, T. J. *et al.* Retinoic acid-induced CD38 expression in HL-60 myeloblastic leukemia cells regulates cell differentiation or viability depending on expression levels. *J. Cell. Biochem.* **97,** 1328–1338 (2006).

33. Nagao, T. *et al.* Proliferation and survival signaling from both Jak2-V617F and Lyn involving GSK3 and mTOR/p70S6K/4EBP1 in PVTL-1 cell line newly established from acute myeloid leukemia transformed from polycythemia vera. *PLoS One* **9,** e84746 (2014).
34. Bunaciu, R. P. *et al.* 6-formylindolo(3,2-b)carbazole(FICZ) modulates the signalsome responsible for RA-induced differentiation of HL-60 myeloblastic leukemia cells. *PLoS One* **10,** e0135668 (2015).
35. Scholl, S. *et al.* Additive effects of PI3-kinase and MAPK activities on NB4 cell granulocyte differentiation: Potential role of phosphatidylinositol 3-kinase γ. *J. Cancer Res. Clin. Oncol.* **134,** 861–872 (2008).

Chapter 6 Foreword

The work in this chapter represents an exploratory foray arising from the opportunity presented by the availability a selective Sirt2 inhibitor, TM, generated by Hui Jing and Lu Zhang of Dr. Hening Lin's laboratory. Knowing that histone deacetylase inhibitors are often overexpressed in cancers and in AML in particular, we probed for effects of treatment with TM and two other Sirt2 inhibitors known to be less specific, AGK2 and BML-266, as well as a Sirt1 inhibitor, EX-527, on differentiation and RA-induced differentiation. As Dr. Andrew Yen's lab is primarily focused on differentiation, we hoped to see effects on the process, but we found that the Sirt1 inhibitor did not appear to alter the process and that the Sirt2 inhibitors all exhibited signs of toxicity concurrent with or before effects on differentiation were apparent. TM was synthesized by Hui Jing and Lu Zhang while the other drugs were purchased commercially and the data shown was generated by Robert MacDonald. As a brief foray, we felt the data was not complete enough to pursue peer-reviewed publication, and since the end result was not what we hoped, we did not use additional time to flesh out the work with, for example, evidence that each drug was able to inhibit its target as anticipated.

Chapter 6: The specific Sirt2 inhibitor TM induces cell death but not differentiation, whereas promiscuous Sirt2 inhibitors AGK2 and BML-266 induce cell death as well as the differentiation marker CD11b in HL-60 acute myeloid leukemia cells

6.1 Abstract

All-*trans* retinoic acid (RA) has been used to treat acute promyelocytic leukemia (APL) for several decades by inducing differentiation of the cancerous cells but has proven less effective in other subtypes of acute myeloid leukemia (AML). Combination therapies, however, show promise in their ability to enhance and expand the efficacy of RA-based differentiation therapy. Histone deacetylase (HDAC) inhibitors are one such class of drugs in clinical trials for use in combination with RA for treatment of non-APL AML. The ability to identify and target the appropriate abnormal HDACs is a key step in harnessing HDAC inhibitors in patients. Inhibition of the HDAC Sirt2 has been shown to enhance RA-induced differentiation in the non-APL AML cell line HL-60 as well as the APL cell line NB4, whereas Sirt1 inhibition had no effect. Highly specific inhibitors for another member of the sirtuin family, Sirt1, are commercially available, but many Sirt2 inhibitors function against other sirtuins as well. Hening Lin's laboratory has recently synthesized and characterized a highly specific inhibitor of Sirt2, TM. Here, we assessed the ability of TM, two commercially available Sirt2 inhibitors, AGK2 and BML-266, and a commercially available Sirt1 inhibitor, EX-527, to enhance RA-induced differentiation of HL-60 cells. EX-527, as anticipated, had no effect on RA-induced differentiation. AGK2 and BML-266, but not TM, enhanced expression of a marker of myeloid differentiation, CD11b. Treatment with each of the three Sirt2 inhibitors, however, resulted in decreased cell density and a corresponding increase in Trypan Blue staining. Our results suggest that

specific Sirt2 inhibition may induce cell death rather than enhancement of RA-induced differentiation in HL-60 cells.

6.2 Introduction

The use of all-*trans* retinoic acid (RA) in the treatment of acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML), hinges upon its ability to induce differentiation of the cancerous cells. Alone, RA is induces remission in over 80% of APL patients, but remission is transient unless combination therapy with anthracyclines or arsenic trioxide, the current standard of care, is employed.[1–5] Combination therapies that may enhance the efficacy and durability of RA treatment not only in APL, but also in other subtypes of AML, where patient outcomes are much worse and RA has historically been much less effective, are therefore of great interest for their potential therapeutic applications.[6–8]

In APL, a common feature in roughly 90% of these cancers is the fusion of the retinoic acid receptor alpha (RARα) and promyelocytic leukemia (PML) proteins to form PML-RARα.[9] As a result, histone deacetylase (HDAC) binding domains from PML are acquired and RARα transcriptional activity is repressed to a greater degree than under normal conditions.[10–12] Typically, physiological levels of RA are able to bind to RARα and cause a release of transcriptional repression and subsequent differentiation of immature granulocytes to neutrophils; however, these levels are not enough to de-repress transcriptional activity in APL and pharmacological intervention is required to induce differentiation.[13–15] The standard form of intervention clinically is RA and arsenic trioxide, as introducing higher doses of RA is able to relieve transcriptional repression and

cotreatment promotes degradation of the PML-RARα fusion protein, resulting in granulocytic differentiation.[5,15–17]

In addition to RA, HDAC inhibitors have also been investigated for potential therapeutic use in both APL and AML.[18] There are four classes of HDACs. Classes I, II, and IV have zinc-dependent active sites, whereas class III HDACs, comprised of the seven members of the sirtuin family, possess NAD^+-dependent activities.[19–21] HDAC inhibitors are able to induce cell death in a variety of cancers, including APL and AML.[22–25] However, there are fewer reports suggesting that they are able to enhance differentiation. Valproic acid and a handful of its derivatives, which act against class I and II HDACs, are one such set of HDAC inhibitors that do promote differentiation of HL-60 cells, an RA-responsive non-APL AML cell line.[26,27] Sodium butyrate, which inhibits class I HDACs, also promotes differentiation of HL-60 cells.[28] As in the case of suberoylanilide hydroxamic acid (SAHA), however, the capacity of HDACs to induce differentiation is often limited by their propensity to cause apoptosis.[21,29,30]

Inhibition of Sirt2, a class III HDAC, is also reported to enhance RA-induced differentiation of HL-60 and NB4 cells, an APL model for inducible differentiation.[31] The same report also demonstrated that inhibition or loss of Sirt1 had no effect on differentiation.[31] Many commercially available Sirt2 inhibitors are known to be somewhat promiscuous, however, often acting against multiple members of the sirtuin family.[32–34]

Here, we compared the effects of one Sirt1 inhibitor, EX-527, two commercially available Sirt2 inhibitors, AGK2 and BML-266, and a highly selective Sirt2 inhibitor synthesized by Hening Lin's laboratory, TM, on RA-induced differentiation of HL-60 cells.[33] As anticipated, we saw no effect of EX-527 on RA-induced differentiation of HL-60 cells.[31]

Treatment with the commercially available Sirt2 inhibitors enhanced CD11b expression but also inhibited cell grown and increased cell death. Treatment with TM inhibited cell growth and increased cell death but did not enhance expression of CD11b. Our results suggest that Sirt2 inhibition may be toxic to HL-60 cells; inhibition with a selective drug leads to cell death before any signs of enhancement of differentiation, while use of less selective agents increases signs of both differentiation and toxicity.

6.3 Materials and methods

6.3-1 Antibodies and reagents

Reagents, unless specified otherwise, were purchased from commercial suppliers in the highest purity available and used as supplied. PE-conjugated CD38 (clone HIT2, catalog number 555460) and APC-conjugated CD11b (clone ICRF44, catalog number 550019) were purchased from Becton Dickinson (Franklin Lakes, NJ). AGK2 and EX-527 were purchased from Sigma (St. Louis, MO) and solubilized in DMSO at 4.6 mM and 1 mM, respectively. BML-266 was purchased from Santa Cruz Biotechnology (Dallas, TX) and solubilized in DMSO at 1 mM. Final DMSO concentrations in media were 0.2% or below. The selective Sirt2 inhibitor, TM, was a generous gift of Hening Lin and was solubilized in absolute ethanol at 25 mM.

6.3-2 Cell culture

HL-60 human myeloblastic leukemia cells derived from original patient isolates, a kind gift of Dr. Robert Gallagher and maintained in the lab, were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (GE Healthcare, Chicago, IL) and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA) in a 5%

CO_2 humidified atmosphere at 37 °C. All-*trans* retinoic acid (RA) (Sigma, St. Louis, MO) was solubilized in absolute ethanol. A final concentration of 1 μM was used.

6.3-3 Flow cytometric phenotypic analysis

Immunostaining for CD11b and CD38 was performed as previously described and analyzed using a Becton Dickinson LSR II flow cytometer.[35] Gating was set to exclude 95% of the untreated wild-type HL-60 samples. Propidium iodide (PI) cell cycle analysis was performed as previously described.[35,36]

6.4 Results

6.4-1 The Sirt1 inhibitor EX-527 did not affect RA-induced differentiation

We tested several concentrations of the Sirt1 specific inhibitor, EX-527, to determine whether it affects RA-induced differentiation of HL-60 cells. Using its published IC_{50} value of 38 nM as a base point, we screened from 20 nM up to 500 nM with no noticeable toxicity.[37] We used several markers of differentiation in our assessment: the early cell surface marker CD38, the late cell surface marker CD11b, G_1/G_0 cell cycle arrest, and cell density. As shown in Figure 6.1, we did not observe any effects on these markers at any of the concentrations tested.

Figure 1: EX-527 phenotypic results

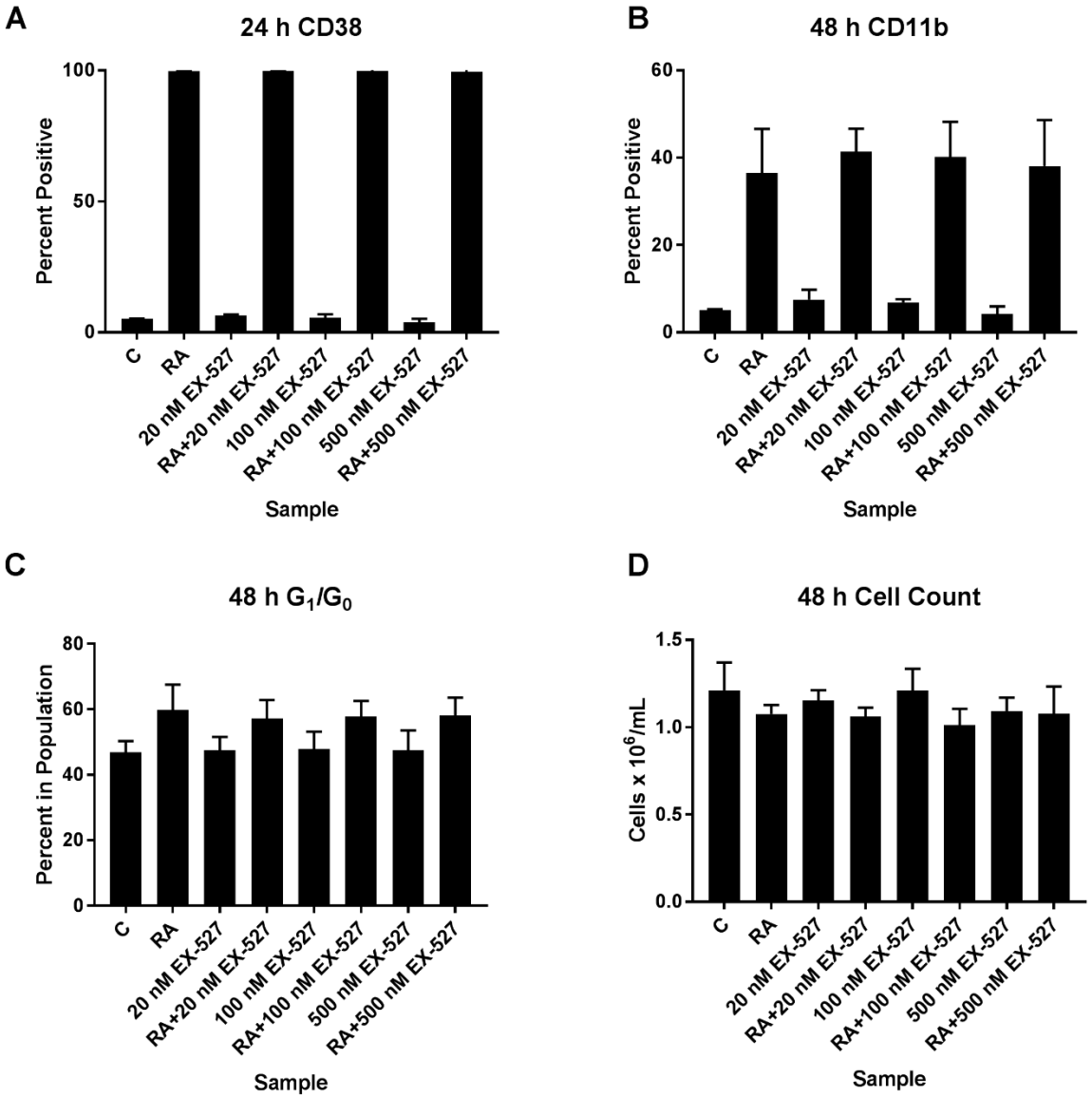


Figure 6.1: EX-527 treatment did not affect RA-induced differentiation. A. HL-60 cells were cultured for 24 h with 1 µM RA or with 20-500 nM EX-527 as indicated and surface expression of CD38 was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. B. HL-60 cells were cultured for 48 h with 1 µM RA or with 20-500 nM EX-527 as indicated and surface expression of CD11b was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. C. Cell cycle distribution, showing the percentage of cells in G_1/G_0, was analyzed by flow cytometry with propidium iodide staining at 48 h. D. Cell density was measured at 48 h using a hemocytometer and 0.2% Trypan Blue exclusion staining. Error bars indicate standard error of the mean (SEM).

6.4-2 Sirt2 inhibitors enhanced RA-induced CD11b expression but also caused cell death

We used several concentrations of three different Sirt2 inhibitors: the commercially available inhibitors AGK2 and BML-266 as well as one synthesized by members of Hening Lin's laboratory, TM. We screened AGK2 around its reported IC_{50} value of 3.5 μM in *in vitro* biochemical assays and in HeLa cells, going from 2 to 5 μM.[38] BML-266 was reported to enhance differentiation in NB4 cells at a concentration of 5 μM, however we observed nearly complete cell death at that level and instead tested from 0.1 to 2 μM.[31] As the IC_{50} of TM in HL-60 cells is roughly 11 μM (Hui Jing, unpublished data), we tested concentrations of 10 and 20 μM.

As shown in Figures 6.2 and 6.3, while we did begin to observe enhanced CD11b expression with the highest doses of AGK2 and BML-266, we also saw decreases in cell density and corresponding increases in levels of cells positive for Trypan Blue staining, an indication of cell death. We observed decreased cell density and increased Trypan Blue staining but not enhancement of CD11b in samples treated with 20 μM TM, as shown in Figure 6.4.

Figure 2: AGK2 phenotypic results

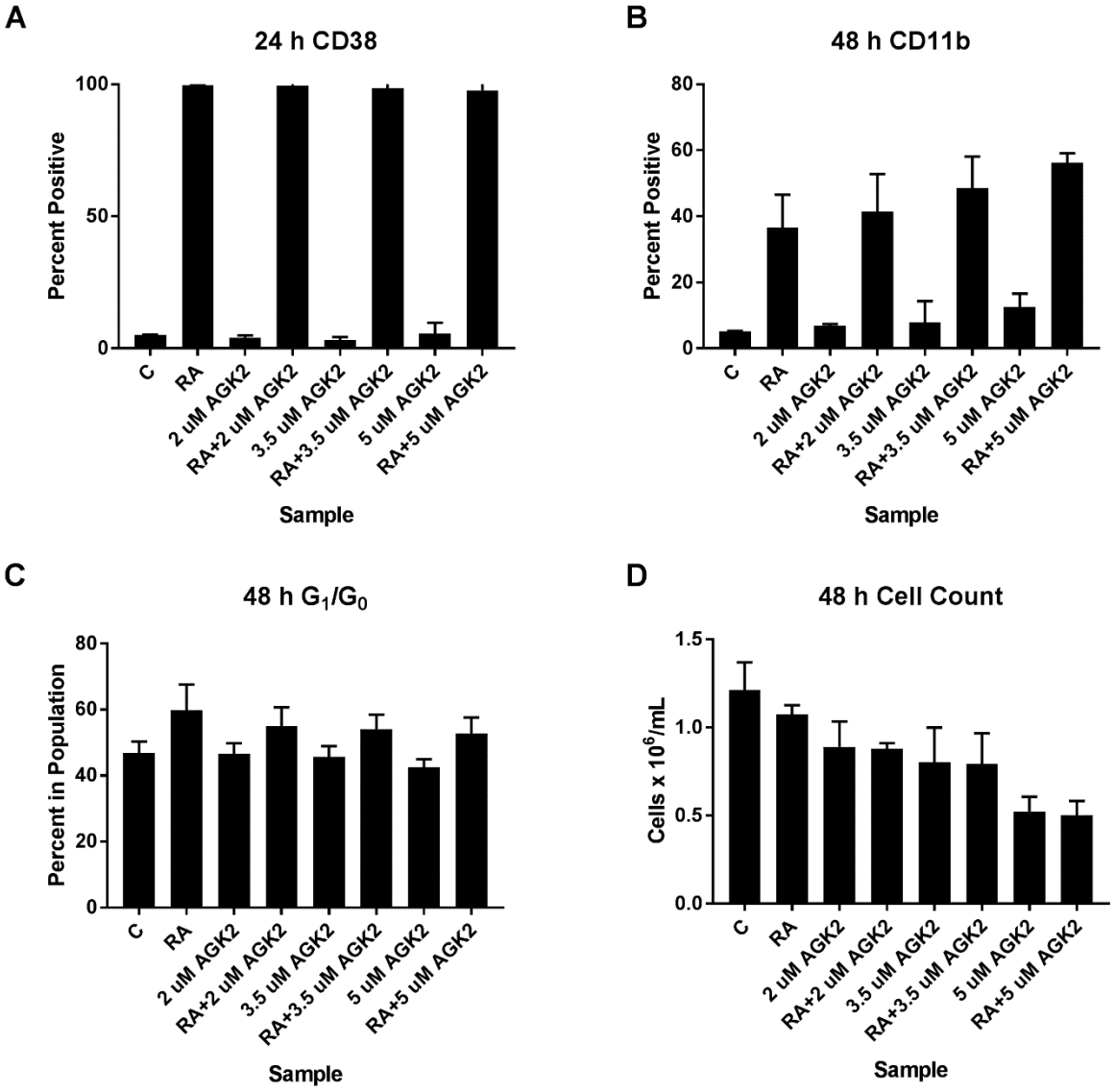


Figure 6.2: AGK2 treatment enhanced RA-induced CD11b expression and inhibited cell growth. A. HL-60 cells were cultured for 24 h with 1 µM RA or with 2-5 µM AGK2 as indicated and surface expression of CD38 was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. B. HL-60 cells were cultured for 48 h with 1 µM RA or with 2-5 µM AGK2 as indicated and surface expression of CD11b was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. C. Cell cycle distribution, showing the percentage of cells in G_1/G_0, was analyzed by flow cytometry with propidium iodide staining at 48 h. D. Cell density was measured at 48 h using a hemocytometer and 0.2% Trypan Blue exclusion staining. Error bars indicate standard error of the mean (SEM).

Figure 3: BML-266 phenotypic results

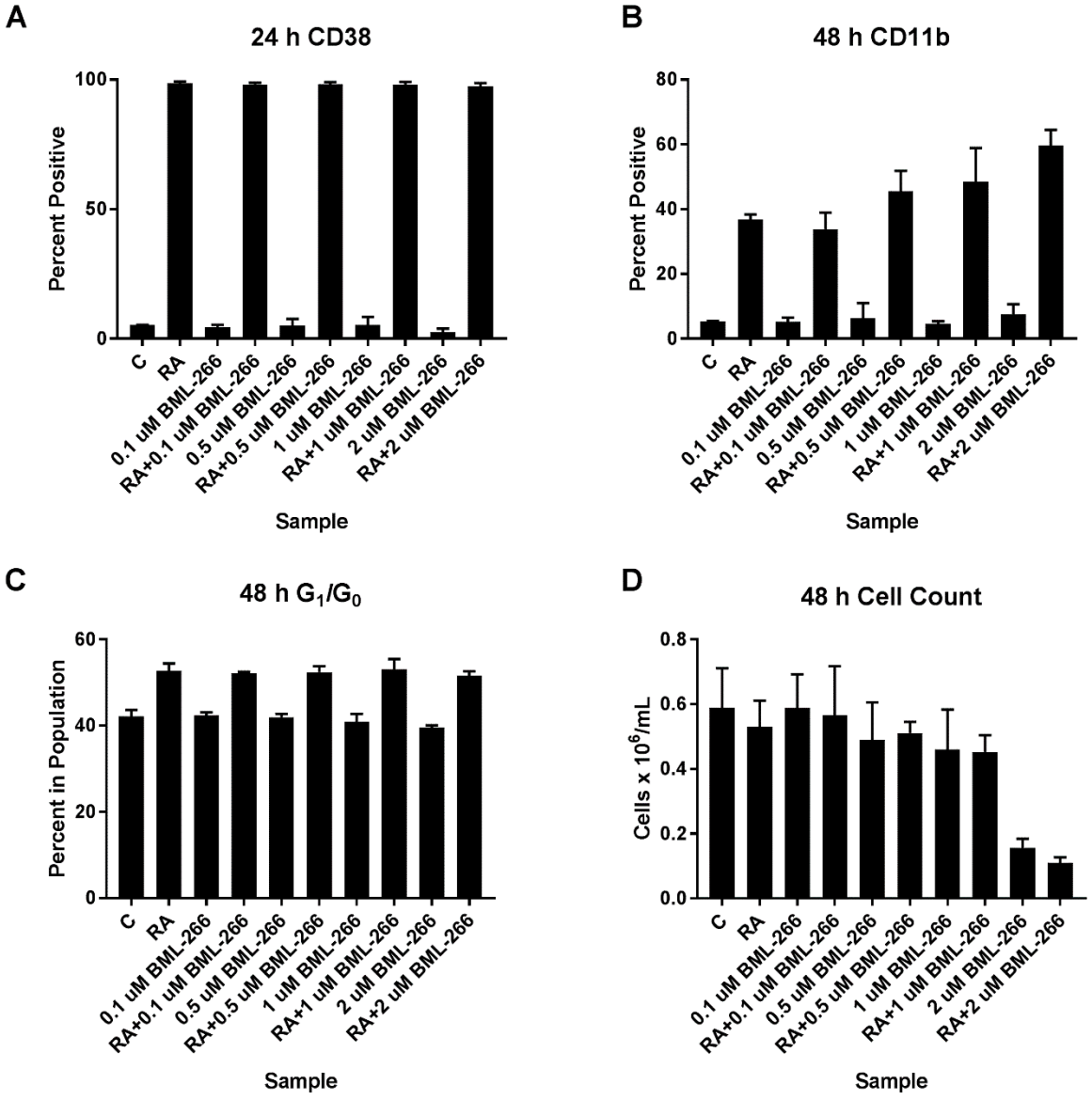


Figure 6.3: BML-266 treatment enhanced RA-induced CD11b expression and inhibited cell growth. A. HL-60 cells were cultured for 24 h with 1 µM RA or with 0.1-2 µM BML-266 as indicated and surface expression of CD38 was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. B. HL-60 cells were cultured for 48 h with 1 µM RA or with 0.1-2 µM BML-266 as indicated and surface expression of CD11b was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. C. Cell cycle distribution, showing the percentage of cells in G_1/G_0, was analyzed by flow cytometry with propidium iodide staining at 48 h. D. Cell density was measured at 48 h using a hemocytometer and 0.2% Trypan Blue exclusion staining. Error bars indicate standard error of the mean (SEM).

Figure 4: TM phenotypic results

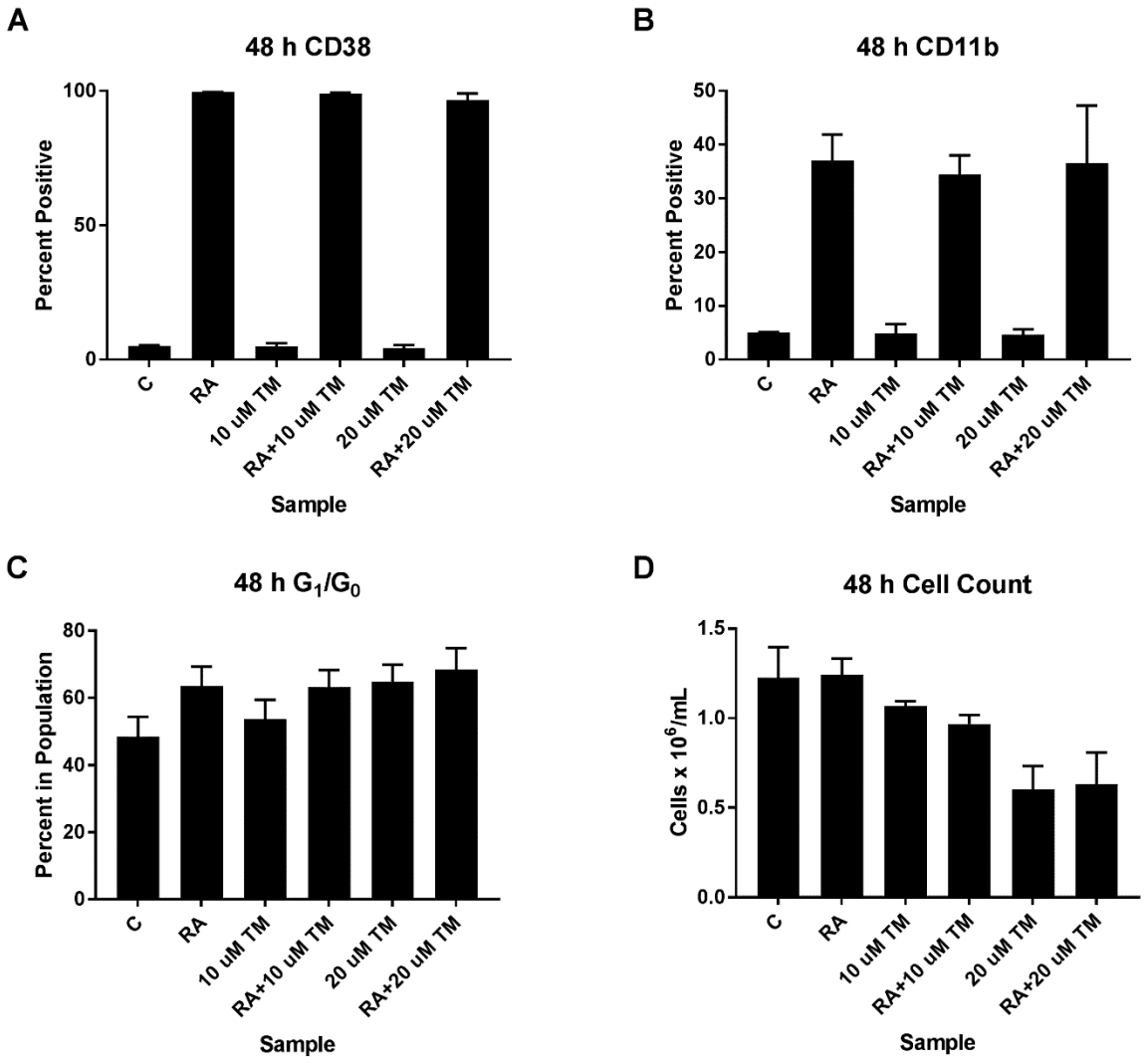


Figure 6.4: TM treatment inhibited growth of HL-60 cells. A. HL-60 cells were cultured for 48 h with 1 μM RA or with 10 or 20 μM TM as indicated and surface expression of CD38 was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. B. Surface expression of CD11b was analyzed by flow cytometry. Gating to discriminate positive cells was set to exclude 95% of untreated controls. C. Cell cycle distribution, showing the percentage of cells in G_1/G_0, was analyzed by flow cytometry with propidium iodide staining at 48 h. D. Cell density was measured at 48 h using a hemocytometer and 0.2% Trypan Blue exclusion staining. Error bars indicate standard error of the mean (SEM).

6.5 Discussion

Current differentiation therapy of APL using RA in combination with arsenic trioxide is highly effective, and response is predicated on the ability of RA to induce differentiation of the cancerous cells. Enhancement of the capability of RA to induce differentiation may benefit patient outcomes not only in APL, but also in non-APL AML. Combination therapies using RA as well as other drugs that may improve its efficacy are of particular interest in non-APL AML, where RA has historically been much less successful and patient outcomes are far less favorable.[6–8]

HDACs are one such targetable component of cancers that have generated excitement for their potential use in combination therapies. Their expression and activity are often aberrant and many HDAC inhibitors are effective at inducing apoptosis in a broad range of cancer cell types, including APL and non-APL AML.[22–25,39] Fewer, however, have been shown to induce or enhance differentiation without inducing apoptosis. HDAC activity is essential for transcriptional regulation in normal cells; to minimize the potential for off-target effects, it is vital to identify the HDACs that may benefit from intervention as well as drugs specific to those HDACs.[39–41] Sirtuins, which comprise the class III HDACs, have drawn particular interest for their roles in cancer as well as aging.[42,43]

Here, we evaluated the ability of several Sirt2 inhibitors, as well as one Sirt1 inhibitor, to enhance RA-induced differentiation of the non-APL AML cell line, HL-60. Many commercially available Sirt2 inhibitors, while effective, are promiscuous, targeting not only Sirt2 but other members of the sirtuin family.[32–34] We compared two commercially available Sirt2 inhibitors, AGK2 and BML-266, with a highly specific Sirt2 inhibitor, TM, graciously provided by Hening Lin's laboratory.[33] Consistent with a previous report, we

saw that Sirt1 inhibition did not affect RA-induced differentiation, while the commercially available Sirt2 inhibitors enhanced CD11b expression, a marker of RA-induced differentiation.[31] AGK2 and BML-266, however, began to display toxicity in the form of diminished cell growth and corresponding increases in Trypan Blue staining concurrently with this enhancement of CD11b expression. Treatment with TM did not result in enhanced CD11b expression, but also began to display toxicity at the highest dose evaluated. Overall, our results suggest that specific inhibition of Sirt2 may be toxic to HL-60 cells, whereas a more broad inhibition of sirtuins may enhance differentiation.

REFERENCES

1. Huang, M. E. *et al.* Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* **72,** 567–572 (1988).
2. Wang, Z. Y. & Chen, Z. Acute promyelocytic leukemia: From highly fatal to highly curable. *Blood* **111,** 2505–2515 (2008).
3. Iland, H. J. *et al.* All-trans-retinoic acid, idarubicin, and IV arsenic trioxide as initial therapy in acute promyelocytic leukemia (APML4). *Blood* **120,** 1570–1580 (2012).
4. Estey, E. *et al.* Use of all-trans retinoic acid plus arsenic trioxide as an alternative to chemotherapy in untreated acute promyelocytic leukemia. *Blood* **107,** 3469–3473 (2006).
5. Coombs, C. C., Tavakkoli, M. & Tallman, M. S. Acute promyelocytic Leukemia: Where did we start, where are we now, and the future. *Blood Cancer J.* **5,** e304 (2015).
6. Belhabri, A. *et al.* All trans retinoic acid in combination with intermediate-dose cytarabine and idarubicin in patients with relapsed or refractory non promyelocytic acute myeloid leukemia: A phase II randomized trial. *Hematol. J.* **3,** 49–55 (2002).
7. Schlenk, R. F. *et al.* Phase III study of all-trans retinoic acid in previously untreated patients 61 years or older with acute myeloid leukemia. *Leukemia* **18,** 1798–1803 (2004).
8. Ma, H. S., Robinson, T. M. & Small, D. Potential role for all- trans retinoic acid in nonpromyelocytic acute myeloid leukemia. *Int. J. Hem. Onc.* **5,** 133–142 (2017).
9. Grimwade, D. & Lo Coco, F. Acute promyelocytic leukemia: a model for the role of molecular diagnosis and residual disease monitoring in directing treatment approach in acute myeloid leukemia. *Leukemia* **16,** 1959–1973 (2002).
10. Grignani, F. *et al.* Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature* **391,** 815–818 (1998).
11. Dellaire, G. & Bazett-Jones, D. P. PML nuclear bodies: Dynamic sensors of DNA damage and cellular stress. *Bioessays* **26,** 963–977 (2004).
12. De Thé, H. & Chen, Z. Acute promyelocytic leukaemia: Novel insights into the mechanisms of cure. *Nat. Rev. Cancer* **10,** 775–783 (2010).
13. He, L. Z. *et al.* Distinct interactions of PML-RARα and PLZF-RARα with co-repressors determine differential responses to RA in APL. *Nat. Genet.* **18,** 126–135 (1998).
14. Altucci, L. & Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat. Rev. Cancer* **1,** 181–193 (2001).
15. Tomita, A., Kiyoi, H. & Naoe, T. Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As2O3) in acute promyelocytic leukemia. *Int. J. Hematol.* **97,** 717–725 (2013).
16. Chen, G. *et al.* In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood* **88,** 1052–1061 (1996).
17. Zhang, X. W. *et al.* Arsenic trioxide controls the fate of the PML-RARα oncoprotein by directly binding PML. *Science.* **328,** 240–243 (2010).
18. Schaefer, E. W. *et al.* A phase 2 study of vorinostat in acute myeloid leukemia.

Haematologica **94,** 1375–1382 (2009).
19. Haberland, M., Montgomery, R. L. & Olson, E. N. The many roles of histone deacetylases in development and physiology: Implications for disease and therapy. *Nat. Rev. Genet.* **10,** 32–42 (2009).
20. Chalkiadaki, A. & Guarente, L. The multifaceted functions of sirtuins in cancer. *Nat. Rev. Cancer* **15,** 608–624 (2015).
21. Ceccacci, E. & Minucci, S. Inhibition of histone deacetylases in cancer therapy: Lessons from leukaemia. *Br. J. Cancer* **114,** 605–611 (2016).
22. He, L. Z. *et al.* Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. *J. Clin. Invest.* **108,** 1321–1330 (2001).
23. Amin, H. M., Saeed, S. & Alkan, S. Histone deacetylase inhibitors induce caspase-dependent apoptosis and downregulation of daxx in acute promyelocytic leukaemia with t(15;17). *Br. J. Haematol.* **115,** 287–297 (2001).
24. Kosugi, H. *et al.* In vivo effects of a histone deacetylase inhibitor, FK228, on human acute promyelocytic leukemia in NOD / Shi-scid/scid mice. *Jpn. J. Cancer Res.* **92,** 529–536 (2001).
25. Bolden, J. E., Peart, M. J. & Johnstone, R. W. Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov.* **5,** 769–784 (2006).
26. Deubzer, H. *et al.* Novel valproic acid derivatives with potent differentiation-inducing activity in myeloid leukemia cells. *Leuk. Res.* **30,** 1167–1175 (2006).
27. Bunaciu, R. P. & Yen, A. Activation of the aryl hydrocarbon receptor ahr promotes retinoic acid-induced differentiation of myeloblastic leukemia cells by restricting expression of the stem cell transcription factor Oct4. *Cancer Res.* **71,** 2371–2380 (2011).
28. Yen, A. & Varvayanis, S. DMSO, sodium butyrate, and TPA induce hypophosphorylation of RB with HL-60 cell differentiation. *In Vitro Cell. Dev. Biol. Anim.* **31,** 164–167 (1995).
29. Vrana, J. A. *et al.* Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. *Oncogene* **18,** 7016–7025 (1999).
30. Eckschlager, T., Plch, J., Stiborova, M. & Hrabeta, J. Histone deacetylase inhibitors as anticancer drugs. *Int. J. Mol. Sci.* **18,** E1414 (2017).
31. Sunami, Y. *et al.* Inhibition of the NAD-dependent protein deacetylase SIRT2 induces granulocytic differentiation in human leukemia cells. *PLoS One* **8,** e57633 (2013).
32. Hu, J., Jing, H. & Lin, H. Sirtuin inhibitors as anticancer agents. *Future Med. Chem.* **6,** 945–966 (2014).
33. Jing, H. *et al.* A SIRT2-selective inhibitor promotes c-Myc oncoprotein degradation and exhibits broad anticancer activity. *Cancer Cell* **29,** 297–310 (2016).
34. Shah, A. A., Ito, A., Nakata, A. & Yoshida, M. Identification of a selective SIRT2 inhibitor and its anti-breast cancer activity. *Biol. Pharm. Bull.* **39,** 1739–1742 (2016).
35. Bunaciu, R. P. *et al.* 6-formylindolo(3,2-b)carbazole (FICZ) modulates the signalsome responsible for RA-induced differentiation of HL-60 myeloblastic leukemia cells. *PLoS One* **10,** e0135668 (2015).

36. Reiterer, G. & Yen, A. Platelet-derived growth factor receptor regulates myeloid and monocytic differentiation of HL-60 cells. *Cancer Res.* **67,** 7765–7772 (2007).
37. Solomon, J. M. *et al.* Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol. Cell. Biol.* **26,** 28–38 (2006).
38. Outeiro, T. F. *et al.* Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science* **317,** 516–519 (2007).
39. Ropero, S. & Esteller, M. The role of histone deacetylases (HDACs) in human cancer. *Mol. Oncol.* **1,** 19–25 (2007).
40. Delcuve, G. P., Khan, D. H. & Davie, J. R. Roles of histone deacetylases in epigenetic regulation: Emerging paradigms from studies with inhibitors. *Clin. Epigenetics* **4,** 5 (2012).
41. Dai, Y. & Faller, D. V. Transcription regulation by class III histone deacetylases (HDACs)-sirtuins. *Transl. Oncogenomics* **3,** 53–65 (2008).
42. Saunders, L. R. & Verdin, E. Sirtuins: Critical regulators at the crossroads between cancer and aging. *Oncogene* **26,** 5489–5504 (2007).
43. O'Callaghan, C. & Vassilopoulos, A. Sirtuins at the crossroads of stemness, aging, and cancer. *Aging Cell* **16,** 1208–1218 (2017).

Chapter 7: Discussion and future directions

The use of all-*trans* retinoic acid (RA) to treat acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML) has transformed patient outcomes; where it was once a virtual death sentence, it is now a highly survivable disease. The standard of care, use of RA in combination with arsenic trioxide, has given patients nearly a 95% remission rate and 5-year disease-free survival rates nearly as high.[1]

The prognosis for non-APL AML patients, however, remains poor by comparison; historically, RA has proven far less effective in non-APL AML than in APL and the traditional chemotherapeutic agents cytarabine and anthracyclines.[2] Remission rates are typically around 60-70%, but the 5-year survival rate is only 27%.[3,4] Patients under age 60 fare much better, with remission rates closer to 80% and 5-year survival rates of about 40%, but the disease primarily affects those over age 65.[2,5] While RA treatment has been tested for use in non-APL AML in various clinical trials, many depict only marginal differences with RA treatment compared to traditional approaches.[6]

AML is a highly heterogeneous disease, however, and as various genetic markers are identified and grouped, there is renewed potential for RA to be effective in AML in combination therapies.[7] The HL-60 cell line, for example, is an AML cell line that responds well to RA treatment and has been used as a model for RA-induced differentiation for several decades.[8–10] Recently, a report from our lab measured responses of about a dozen samples of primary AML cells and the HL-60 cell line to treatment with RA, either alone or in combination with the Src family kinase inhibitor (SFK) bosutinib or the aryl hydrocarbon receptor agonist 6-formylindolo(3,2-b) carbazole.[11] The report indicated that the response of HL-60 grouped closely with that of a particular primary sample with wild-

type NPM1, low CD34 expression, and had c-Cbl, Lyn, and c-Raf expression that could be regulated by RA.[11] This may represent a previously uncharacterized subtype of AML in which RA could be an effective treatment.

For the best chances of RA to benefit AML patients, whether directly or indirectly, we need a deeper understanding of its mechanism of action in the context of inducible differentiation. In a basic sense, RA is known to drive differentiation via its interactions with the RA receptors (RARs) and retinoid X receptors (RXRs).[12–17] RAR/RXR heterodimers act as transcriptional regulators, binding at specific sites of the genome known as RA response elements (RAREs).[17] Without RA, the RAR/RXR heterodimers bound at RAREs hold the affected genes in transcriptionally repressed states, and when RA is present, it is able to remove this transcriptional repression.[12,17] Over 500 genes are known to be regulated by RA, but since RA has numerous biological roles, it is unlikely that modulation of all of its gene targets is necessary for differentiation.[17] Therefore, identification of the key elements regulated by RA essential for the differentiation process would provide candidates for therapeutic targeting.

In probing for RA-regulated elements required for differentiation, one signaling pathway in particular has stood out: a mitogen-activated protein kinase (MAPK) cascade comprised of c-Raf, MEK, and ERK. Activation of this signaling cascade is required for RA-induced differentiation of HL-60 cells; inhibition of c-Raf or MEK has been shown to cripple RA-induced differentiation, while overexpression of c-Raf enhances the process.[18–22] Activation of the signaling pathway is durable, lasting 48 – 72 h, and is first detectable 12 – 24 h after RA treatment.[20] Knowing that the MAPK cascade is essential for RA-induced differentiation but does not occur immediately following RA treatment, our

attention shifted to other proteins upregulated by RA on an earlier timescale that may serve as initiating factors for the cascade.

CD38, an ectoenzyme receptor, exhibits a number of characteristics that suggested it may be an essential driver of RA-induced differentiation. The protein exhibits very low basal expression levels, but its expression is starkly upregulated shortly after RA treatment.[23,24] Previous reports have shown that ectopic expression of CD38 can enhance RA-induced differentiation while siRNA and morpholino antisense oligonucleotides targeting CD38 inhibit the process.[24,25] The enzymatic activity of CD38, converting nicotinamide adenine dinucleotide (NAD^+) to adenosine diphosphate ribose (ADPR) and cyclic ADPR, is not necessary for differentiation, suggesting that its receptor activity may be important.[26] Ligation of CD38 using its ligand, CD31, or anti-CD38 monoclonal antibodies induces a short-lived activation of several downstream proteins including c-Raf.[27–31]

Probing for ways in which CD38 may be tied to differentiation-driving signaling pathways such as the MAPK axis, we found that CD38 is able to interact with two cytosolic signaling regulators, Vav1 and SLP-76, as well as the Src family kinase (SFK) Lyn.[32] We also show that these proteins interact with one another.[32] This implies that pathways regulated by these proteins may in turn be modulated by CD38 and one another.[32] In relation to the previously mentioned MAPK signaling axis, murine dendritic cells and Jurkat T cells deficient in SLP-76 exhibit impaired activation of ERK.[33,34] Vav1 is an essential component of RA-induced differentiation, as knockdown impairs the process while overexpression enhances it.[35] Lyn is the predominantly active SFK in HL-60 cells, and

although its specific role in regulation of MAPK signaling is not well understood, SFKs are known to positively modulate it as well as to contribute to cell transformation.[36–38]

Knowing that SFKs are involved, we also test and demonstrate differential effects of the SFK inhibitor, PP2, on downstream signaling with and without CD38 ligation with the monoclonal antibody IB4.[32] When treated with PP2 only for 48 h, Lyn activation is inhibited, and when treated with RA only for 48 h, it is enhanced.[32] Concurrent 48 h treatment with RA and PP2 preserves Lyn activity; however, when PP2 is added late, during the final hour of the 48 h treatment period, Lyn activity is inhibited.[32] Interestingly, concurrent treatment with RA and dasatinib, a clinically relevant SFK inhibitor used in treatments of chronic myeloid leukemia and acute lymphocytic leukemia, favors inhibition of Lyn activity.[32,39,40] As Lyn expression is upregulated following co-treatment with RA and either SFK inhibitor, it is possible that Lyn may enhance differentiation by serving as a scaffold. Src in particular has been implicated as a scaffold, and other kinases such as c-Raf and ERK are known to have roles as scaffolds as well.[41–45] CD38 ligation with IB4 shows a similar trend in terms of activation of both the c-Cbl adapter and the MAPK p85 PI3K; ligation drives these signals, and concurrent treatment with RA and PP2 preserves them while late treatment with PP2 cripples them.[32] Following the trend, we also saw that interactions between many of these partners, Vav1 and CD38, Vav1 and Lyn, SLP-76 and CD38, p85 PI3K and CD38, and p85 PI3K and Lyn, were enhanced with concurrent RA and PP2 treatment followed by IB4 stimulation, while late PP2 treatment diminished them.[32]

These results added to the pre-existing literature suggesting a dependence of RA-induced differentiation on CD38 and suggested that Lyn kinase activity may be important

for the process through regulation of CD38-driven activity. Ligation of CD38 drove p85 PI3K phosphorylation as well as c-Cbl phosphorylation, a feature of RA-induced differentiation.[28,31,32] While PP2 inhibition is not specific to Lyn, previous reports have demonstrated that Lyn is the major SFK present and active in HL-60 cells.[36,37] Our findings suggest that Lyn, therefore, may also play an important role in RA-induced differentiation, since inhibition crippled various interactions between signaling partners as well as CD38-driven c-Cbl and p85 PI3K phosphorylation.

With this additional data implicating CD38 as an early initiator of RA-induced differentiation, and with a previous report discounting its enzymatic activity in the process, we turned to investigate its receptor function.[26] Since antibody-mediated ligation induced phosphorylation of the downstream partners c-Cbl and p85 PI3K, we used a synthetic linker based on the suicide substrate, F-araNAD$^+$, dimeric F-araNAD$^+$ (dF-araNAD$^+$), to induce homodimerization of the protein and probe for phenotypic effects.[27–31,46,47] Dimerization is a common mechanism of membrane receptor signaling, and CD38 has been reported to form homodimers in B lymphocytes.[48,49] We made the conjecture that there may be an enhancement of RA-induced differentiation if CD38 generates a downstream signal by homodimerization, and that it may be impaired if it signals via heterodimerization.

Using both western blot and flow cytometry-based Förster resonance energy transfer (FRET), we demonstrated that treatment with dF-araNAD$^+$ was able to significantly enhance CD38 homodimerization.[50] We added dF-araNAD$^+$ to dimerize CD38 at 8 h or 24 h after RA treatment, testing whether early versus later CD38 dimerization may affect the differentiation process.[50] As a control, we also tested the effect of F-araNAD$^+$.[50]

Neither had any phenotypic effects on differentiation, suggesting that dimerization may not be important for the process.[50]

Since there are many caveats to these experiments probing for effects of linker-induced CD38 homodimerization, which have been previously outlined in Chapter 3 of this dissertation, we opted to pursue the effects of loss of CD38 on RA-induced differentiation. Using the CRISPR/Cas9 system, we generated three stable HL-60 cell lines in an attempt to delete CD38.[50] Although we did not fully delete the protein, we were able to severely truncate it such that, in the most dramatic instance, only the first 6-7 amino acids of CD38 were conserved to generate a peptide 15 amino acids long prior to a stop codon.[50] In the other two lines, we characterized less extreme truncations, such that only the first 45-50 amino acids remained to generate products no longer than 62 residues long of what is normally a 300-residue long protein.[50] Additionally, nearly the entire extracellular domain, comprising residues 43-300, was deleted in every case, leaving only the complete cytoplasmic domain (residues 1-20) and transmembrane domain (residues 21-42) in two of the three CRISPR-derived cell lines.[50] We confirmed deletions via western blot and flow cytometry to demonstrate that the antibodies could no longer detect CD38 in the CRISPR-derived cells, through PCR to determine the sequences of the intact sections of the gene, and finally through the NGD^+ enzymatic assay.[50] This fluorometric assay shows that enzymatic activity of CD38, which depends on the extracellular domain, was crippled in the CRISPR-derived cell lines.[50]

While we did not generate any cell lines with CD38 entirely deleted, we did create three lines expressing severely truncated forms of the protein; two that included the cytosolic and transmembrane domains and one that included only a portion of the cytosolic

domain.[50] We assessed the phenotypic effects of RA treatment, anticipating RA-induced differentiation to be crippled given the body of circumstantial evidence previously presented. We were surprised to find the cells' ability to differentiate in response to RA treatment intact.[50] Additionally, we probed for changes in levels of a subset of signaling molecules important for the differentiation process, including MEK, phosphorylated MEK, and ERK, and found no significant changes in their expression in the CRISPR-derived cell lines compared to HL-60 cells regardless of RA treatment.[50] Taken together, our results strongly suggest that CD38 is not essential for RA-induced differentiation, with the possible caveat that the small cytosolic peptide remaining in one of the lines may be enough to drive the process.

Having demonstrated surprisingly that CD38 expression was not necessary for differentiation, we turned our attention to another receptor, one which is known to be required for RA-induced differentiation, CXCR5. CXCR5 is a G-protein coupled receptor best characterized in the context of naïve B cells and T follicular helper cells, where it helps to promote maturation of the cells and formation of germinal centers.[51–55] These cells highly express CXCR5, which directs migration toward its ligand, CXCL13, produced by stromal cells in lymph nodes.[56,57] In HL-60 cells, deletion of the protein via homologous recombination cripples RA-induced differentiation, whereas ectopic expression has been shown to enhance it.[22,58] Expression of CXCR5 mRNA is induced following RA treatment, though induction is relatively slow and does not peak until 48-72 h after treatment.[58] CXCR5 ectopic expression enhances signaling along the c-Raf/MEK/ERK axis, and downregulation of c-Raf via siRNA or inhibition of c-Raf or MEK blocked RA-induced CXCR5 mRNA production, suggesting a link between the MAPK axis and CXCR5.[22,58]

We sought to characterize this link, probing whether dimerization of CXCR5 may explain downstream signaling along the MAPK axis to drive differentiation. While few studies detail specific CXCR5 interaction partners other than CXCL13, we did find papers reporting CXCR5 homodimerization, heterodimerization with CXCR4, and heterodimerization with EBI2.[59,60] Intending to use these as positive controls, we also screened for interaction between CXCR5 and two membrane proteins upregulated in RA-induced differentiation, CD38 and CD11b, as well as two cytosolic proteins implicated in the process, c-Cbl and Lyn.

To accomplish this, we created HL-60 transfectants that stably overexpressed CXCR5 ($CXCR5^+$) and performed an initial migration assay to confirm that the protein was active. CXCR5 is not expressed in HL-60 cells until treated with RA, and comparing the protein levels using flow cytometry, we found that expression levels even in RA-treated cells were quite low compared to the $CXCR5^+$ cells and Raji cells, a positive control. Both RA-treated HL-60 cells and untreated $CXCR5^+$ cells, however, did demonstrate migration toward CXCL13, indicating that the ectopically expressed protein was functionally active. Interestingly, migration followed a bell-shaped curve, with a moderate dose eliciting the strongest response.

We probed for CXCR5 interaction partners using a similar FRET procedure we tested and confirmed in our CD38 linker experiments.[50] In this case, however, we used two slightly more sensitive dyes that should have allowed for a better limit of detection. Surprisingly, we were unable to detect a FRET signal between CXCR5 and any of our candidates (CD11b, CD38, CXCR4, CXCR5, EBI2, c-Cbl, and Lyn) in HL-60 and $CXCR5^+$ cells with and without RA treatment. The candidate binding partners may not interact with

CXCR5 in the context of HL-60 cells or they may interact as part of a larger complex that may inhibit detection via FRET.

Additionally, we tested the phenotypic shift and MAPK signaling attributes of our CXCR5^{+} cell line in response to RA treatment but found no difference between CXCR5^{+} and HL-60 cells. This may be due to the degree of CXCR5 expression or differences in serum CXCL13 levels compared to previous reports in which CXCR5 ectopic expression led to enhanced RA-induced differentiation.

Future studies could optimize the phenotypic dose-response between levels of CXCR5 expression and enhancements in RA-induced differentiation as well as determine the influence of CXCL13 levels on differentiation. A stably transfected cell line expressing CXCR5 at a level similar to that of HL-60 cells treated with RA could be a good starting point. The CXCL13-mediated migration data indicate that there is an optimal dose required for migration. Given the direct relationship previously observed between CXCR5 expression and activation of the c-Raf/MEK/ERK axis, it is plausible that MAPK signaling may be driven in part by CXCL13-mediated stimulation of CXCR5.[22,58] There could be a similar optimal dose for stimulation of CXCR5-mediated signaling that may in turn drive activation of the c-Raf/MEK/ERK axis and thus differentiation. Adding a degree of merit to this conjecture, some G-protein coupled receptors are known to be able to drive ERK signaling through various mechanisms including SFK and PI3K activation, both of which occur in RA-induced differentiation of HL-60 cells.[61–63]

Apart from probing single protein targets for their potential relevance in RA-induced differentiation, another path to set the foundation for differentiation therapy in non-APL AML is through testing combination therapeutics. Two particular SFK inhibitors, PP2 and

dasatinib, have previously shown promise in their ability to enhance RA-induced differentiation of HL-60 cells.[36,37,64] Both of these inhibitors, however, are relatively broad inhibitors of tyrosine kinases, and although dasatinib is clinically approved for use in acute lymphocytic leukemia and chronic myelogenous leukemia, it is often poorly tolerated by patients due to its toxicity.[65–68]

We performed an initial characterization of HL-60 treatment with RA alone versus combined treatment with RA and bosutinib, another SFK inhibitor approved for clinical use in the treatment of chronic myelogenous leukemia that is attractive due to its lower toxicity profile.[68,69] Similarly to dasatinib, we found that bosutinib enhanced RA-induced differentiation.[37,64,69] Bosutinib treatment also diminished Lyn phosphorylation and caused an increase in Lyn and Fgr expression, matching previous data for dasatinib co-treatments; enhanced expression could be a compensatory response as activity of the proteins is blocked.[64,69] We also observed enhancements of c-Raf phosphorylation, consistent with the dasatinib data.[64,69] Additionally, we assessed expression of c-Cbl and mTOR, which were decreased with bosutinib treatment, and expression of AhR and p85 PI3K, which were enhanced.[69] While the decrease of c-Cbl expression was surprising, since enhanced expression is associated with enhanced RA-induced differentiation, other changes in protein expression were consistent with our proposed paradigm of signalsome-originated signaling driving RA-induced differentiation.[70] AhR, for example, is activated in response to PP2 and here we saw enhanced expression of the protein.[71] Activity of p85 PI3K is enhanced in RA-induced differentiation, while mTOR is hypothesized to be activated by Lyn, which is inhibited by bosutinib.[72,73]

Based on previous reports with PP2 and dasatinib as well as our current work on bosutinib, SFK inhibitors represent an intriguing class of drugs worthy of further investigation for potential use in combination with RA in AML. Bosutinib is particularly attractive given its current clinical use and relatively benign toxicity profile. Future work could involve kinome profiling of bosutinib to ensure its specificity against SFKs as opposed to tyrosine kinases broadly. Further characterization of the role of Lyn in RA-induced differentiation may prove beneficial as well. It is noteworthy that Lyn expression is enhanced following co-treatment with PP2, dasatinib, or bosutinib and RA, but inhibition is only seen with dasatinib and bosutinib and RA; co-treatment with PP2 and RA enhances its activity.[64,69] Rather than its activity, Lyn's potential function as a scaffold may be the mechanism by which these SFK inhibitors enhance RA-induced differentiation. Such a function would be in line with prior reports suggesting that Src has scaffolding functions, and other kinases such as c-Raf and ERK have been shown to act as scaffolds in certain cases.[41–45]

Another class of inhibitors that have demonstrated potential to be used in combination therapy with RA are histone deacetylase (HDAC) inhibitors. Four are currently approved for clinical use in various blood cancers in the USA, but three of these are pan-HDAC inhibitors and one broadly targets class I HDACs.[74] Due to the essential role HDACs play in maintaining normal gene expression, this presents the opportunity for more selective HDAC inhibitors to be utilized as aberrant HDAC activity is pinpointed. Various HDAC inhibitors have been shown in induce apoptosis in cancer cells, including AML cells, and several have also been shown to enhance RA-induced differentiation, suggesting they may be attractive for use in AML.[75–81]

We performed a series of experiments to explore the phenotypic effects of several sirtuin inhibitors, comparing effects of a specific Sirt1 inhibitor and less specific inhibitors of Sirt2 to a highly specific inhibitor Sirt2 inhibitor synthesized by collaborators in Dr. Hening Lin's lab.[82–84] A previous study demonstrated enhancement of RA-induced differentiation in an APL cell line, NB4, as well as HL-60 cells, with Sirt2 inhibition or knockdown but not Sirt1 inhibition or knockdown.[85] Interestingly, our results matched their findings using the Sirt1-specific inhibitor, EX-527, but we observed that treatment with the broad Sirt2 inhibitors, AGK2 and BML-266, resulted in enhancements of the differentiation marker CD11b with concurrent increases in cell death. Inhibition using the specific Sirt2 inhibitor from Hening Lin's lab, TM, resulted in cell death before any enhancement of RA-induced differentiation became apparent. Each of the Sirt2 inhibitors began to exhibit toxicity at the highest doses assessed regardless of RA treatment.

Although our results indicated that these Sirt2 inhibitors were not able to induce differentiation or enhance RA-induced differentiation without signs of toxicity, Sirt2 inhibition may still be beneficial to patients. Two recent reports indicated that high levels of Sirt2 in AML were associated with unfavorable patient outcome and relapsed patients expressed higher levels of Sirt2 than new patients, suggesting that this may be the case.[86,87] One of these reports also demonstrated that in HL-60 cells, Sirt2 expression is directly related to expression of the multidrug resistance protein MRP1 and suggests that Sirt2 enhances MRP1 expression in an ERK-dependent manner.[87] Activation of ERK, however, is also a feature of RA-induced differentiation; future work may focus on these roles that lead to such divergent outcomes.

The work presented in this dissertation discounts CD38 as a key activator of RA-induced differentiation and also eliminates certain candidate mechanisms by which CXCR5 drives the process but adds to the growing body of literature demonstrating the potential of combination therapy to be applied in the treatment of non-APL AML. A brief summary figure of some of the proteins examined in this dissertation in the context of RA-induced differentiation is shown in Figure 7.1. Additional studies are needed to determine the role of CXCR5 and its relation to MAPK signaling and RA-induced differentiation. Historically, clinical trials using RA in the treatment of non-APL AML have not shown dramatic results like those seen in APL.[6] Due to the heterogeneity of AML, however, it is possible that effective therapies that include RA are limited to certain subtypes of the cancer, and a recent study from our laboratory shows that the non-APL AML RA-responsive HL-60 cell line may represent such a subtype.[7,11] Slowly but surely, the field of cancer biology is evolving to make practical use of the reams of data we are able to gather from primary samples.[88] Cancer treatment strategies are frequently approached on a case-by-case basis, and the future of cancer treatment looks to become even more intensely personalized, with targeted therapies based on individual characteristics and subtleties of each patient's disease leading to milder treatments and better outcomes for all.[88,89]

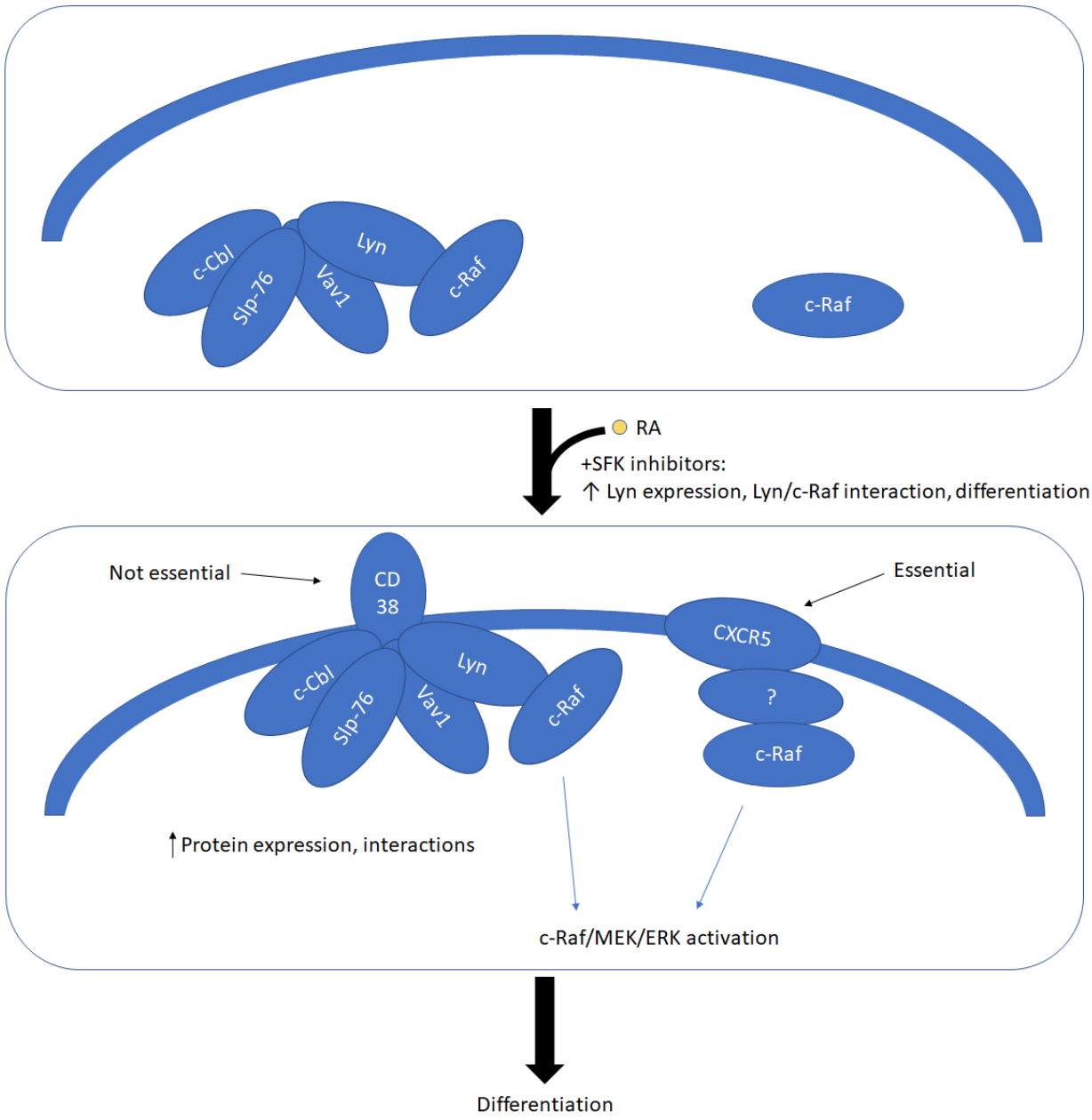

c-Cbl
Slp-76
Lyn
Vav1
c-Raf
c-Raf
RA
+SFK inhibitors:
↑ Lyn expression, Lyn/c-Raf interaction, differentiation
Not essential
CD 38
Essential
CXCR5
?
c-Raf
c-Cbl
Slp-76
Lyn
Vav1
c-Raf
↑Protein expression, interactions
c-Raf/MEK/ERK activation
Differentiation

Figure 7.1: Brief summary of RA-induced differentiation showing roles of proteins examined in this dissertation. A putative complex involving c-Cbl, Slp-76, Vav1, and Lyn connected to c-Raf is able to interact, and expression and interactions are enhanced following RA treatment. CD38 and CXCR5 are expressed following RA treatment and members of the complex interact with CD38. CD38 can enhance downstream c-Raf activation and subsequent differentiation, but CD38 is not essential for the process. CXCR5, however, is necessary for driving c-Raf activation and differentiation, but the manner in which it is linked to c-Raf is unknown. Addition of SFK inhibitors concurrent with RA treatment enhances expression of Lyn, the interaction of Lyn and c-Raf, c-Raf/MEK/ERK activation, and differentiation.

REFERENCES

1. McCulloch, D., Brown, C. & Iland, H. Retinoic acid and arsenic trioxide in the treatment of acute promyelocytic leukemia: Current perspectives. *Onco Targets Ther.* **10,** 1585–1601 (2017).
2. Dombret, H. & Gardin, C. An update of current treatments for adult acute myeloid leukemia. *Blood* **127,** 53–61 (2016).
3. Levy, A. S. & Stump-Sutliff, K. Acute myeloid leukemia (AML): Your chances for recovery (Prognosis). *University of Rochester Medical Center*
4. Treatment response rates for acute myeloid leukemia. *American Cancer Society* (2016).
5. De Kouchkovsky, I. & Abdul-Hay, M. Acute myeloid leukemia: A comprehensive review and 2016 update. *Blood Cancer J.* **6,** e441 (2016).
6. Ma, H. S., Robinson, T. M. & Small, D. Potential role for all- trans retinoic acid in nonpromyelocytic acute myeloid leukemia. *Int. J. Hem. Onc.* **5,** 133–142 (2017).
7. Li, S., Mason, C. E. & Melnick, A. Genetic and epigenetic heterogeneity in acute myeloid leukemia. *Curr. Opin. Genet. Dev.* **36,** 100–106 (2016).
8. Collins, S. J., Gallo, R. C. & Gallagher, R. E. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* **270,** 347–349 (1977).
9. Fontana, J. A., Colbert, D. A. & Deisseroth, A. B. Identification of a population of bipotent stem cells in the HL60 human promyelocytic leukemia cell line. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3863–3866 (1981).
10. Dalton, W. T. *et al.* HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood* **71,** 242–247 (1988).
11. Bunaciu, R. P. *et al.* Potential for subsets of wt-NPM1 primary AML blasts to respond to retinoic acid treatment. *Oncotarget* **5,** 4134–4149 (2017).
12. Altucci, L. & Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat. Rev. Cancer* **1,** 181–193 (2001).
13. Lazar, M. Nuclear Receptor Corepressors. *Nucl. Recept. Signal.* **1,** e001 (2003).
14. Chekravarti, D. *et al.* Role of CBP/P300 in nuclear receptor signalling. *Nature* **383,** 99–103 (1996).
15. Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. & Evans, R. M. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345,** 224–229 (1990).
16. Mangelsdorf, D. J. *et al.* Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6,** 329–344 (1992).
17. Balmer, J. E. & Blomhoff, R. Gene expression regulation by retinoic acid. *J. Lipid Res.* **43,** 1773–1808 (2002).
18. Yen, A, Roberson, M. S., Varvayanis, S. & Lee, A. T. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res.* **58,** 3163–3172 (1998).
19. Yen, A. & Varvayanis, S. Retinoic acid increases amount of phosphorylated RAF; ectopic expression of cFMS reveals that retinoic acid-induced differentiation is more strongly dependent on ERK2 signaling than induced GO arrest is. *In Vitro Cell. Dev. Biol. Anim.* **36,** 249–255 (2000).

20. Hong, H. Y., Varvayanis, S. & Yen, A. Retinoic acid causes MEK-dependent RAF phosphorylation through RARα plus RXR activation in HL-60 cells. *Differentiation* **68,** 55–66 (2001).
21. Miranda, M. B., McGuire, T. F. & Johnson, D. E. Importance of MEK-1/2 signaling in monocytic and granulocytic differentiation of myeloid cell lines. *Leukemia* **16,** 683–692 (2002).
22. Wang, J. & Yen, A. A MAPK-positive feedback mechanism for BLR1 signaling propels retinoic acid-triggered differentiation and cell cycle arrest. *J. Biol. Chem.* **283,** 4375–4386 (2008).
23. Kishimoto, H. *et al.* Molecular mechanism of human CD38 gene expression by retinoic acid. Identification of retinoic acid response element in the first intron. *J. Biol. Chem.* **273,** 15429–15434 (1998).
24. Lamkin, T. J. *et al.* Retinoic acid-induced CD38 expression in HL-60 myeloblastic leukemia cells regulates cell differentiation or viability depending on expression levels. *J. Cell. Biochem.* **97,** 1328–1338 (2006).
25. Munshi, C. B., Graeff, R. & Lee, H. C. Evidence for a causal role of CD38 expression in granulocytic differentiation of human HL-60 cells. *J. Biol. Chem.* **277,** 49453–49458 (2002).
26. Congleton, J., Jiang, H., Malavasi, F., Lin, H. & Yen, A. ATRA-induced HL-60 myeloid leukemia cell differentiation depends on the CD38 cytosolic tail needed for membrane localization, but CD38 enzymatic activity is unnecessary. *Exp. Cell Res.* **317,** 910–919 (2011).
27. Deaglio, S. *et al.* Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J. Immunol.* **160,** 395–402 (1998).
28. Kontani, K. *et al.* Tyrosine phosphorylation of the c-cbl proto-oncogene product mediated by cell surface antigen CD38 in HL-60 cells. *J. Biol. Chem.* **271,** 1534–1537 (1996).
29. Zubiaur, M., Izquierdo, M., Terhorst, C., Malavasi, F. & Sancho, J. CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-ζ/ζ-associated protein-70 signaling pathways in Jurkat T lymphocytes. *J. Immunol.* **159,** 193–205 (1997).
30. Zubiaur, M. *et al.* CD38 is associated with lipid rafts and upon receptor stimulation leads to Akt/protein kinase B and Erk activation in the absence of the CD3-zeta immune receptor tyrosine-based activation motifs. *J. Biol. Chem.* **277**, 13-22 (2001).
31. Silvennoinen, O. *et al.* CD38 signal transduction in human B cell precursors. Rapid induction of tyrosine phosphorylation, activation of syk tyrosine kinase, and phosphorylation of phospholipase C-gamma and phosphatidylinositol 3-kinase. *J. Immunol.* **156,** 100–107 (1996).
32. Congleton, J., Shen, M., MacDonald, R., Malavasi, F. & Yen, A. Phosphorylation of c-Cbl and p85 PI3K driven by all-trans retinoic acid and CD38 depends on Lyn kinase activity. *Cell. Signal.* **26,** 1589–1597 (2014).
33. Luckashenak, N. A., Ryszkiewicz, R. L., Ramsey, K. D. & Clements, J. L. The Src homology 2 domain-containing leukocyte protein of 76-kDa adaptor links integrin ligation with p44/42 MAPK phosphorylation and podosome distribution in murine dendritic cells. *J. Immunol.* **177,** 5177–5185 (2006).

34. Yablonski, D., Kuhne, M. R., Kadlecek, T. & Weiss, A. Uncoupling of nonreceptor tyrosine kinases from PLC-1 in an SLP-76-deficient T cell. *Science* **281,** 413–416 (1998).
35. Bertagnolo, V. *et al.* Vav promotes differentiation of human tumoral myeloid precursors. *Exp. Cell Res.* **306,** 56–63 (2005).
36. Dos Santos, C. *et al.* A critical role for Lyn in acute myeloid leukemia. *Blood* **111,** 2269–2279 (2008).
37. Kropf, P. L., Wang, L., Zang, Y., Redner, R. L. & Johnson, D. E. Dasatinib promotes ATRA-induced differentiation of AML cells. *Leukemia* **24,** 663–665 (2010).
38. Kim, M. P., Park, S. I., Kopetz, S. & Gallick, G. E. Src family kinases as mediators of endothelial permeability: Effects on inflammation and metastasis. *Cell Tissue Res.* **335,** 249–259 (2009).
39. Keskin, D., Sadri, S. & Eskazan, A. E. Dasatinib for the treatment of chronic myeloid leukemia: Patient selection and special considerations. *Drug Des. Devel. Ther.* **10,** 3355–3361 (2016).
40. Foà, R. *et al.* Dasatinib as first-line treatment for adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* **118,** 6521–6528 (2011).
41. Brown, M. T. & Cooper, J. A. Regulation, substrates and functions of src. *Biochim. Biophys. Acta.* **1287,** 121–149 (1996).
42. Kaplan, K. B., Swedlow, J. R., Morgan, D. O. & Varmus, H. E. c-Src enhances the spreading of src-/-fibroblasts on fibronectin by a kinase-independent mechanism. *Genes Dev.* **9,** 1505–1517 (1995).
43. An, S. *et al.* Raf-interactome in tuning the complexity and diversity of Raf function. *FEBS J.* **282,** 32–53 (2015).
44. Advani, S. J. *et al.* Kinase-independent role for CRAF-driving tumour radioresistance via CHK2. *Nat. Commun.* **6,** 8154 (2015).
45. Hong, S. K., Yoon, S., Moelling, C., Arthan, D. & Park, J. I. Noncatalytic function of ERK1/2 can promote Raf/MEK/ERK-mediated growth arrest signaling. *J. Biol. Chem.* **284,** 33006–33018 (2009).
46. Sleath, P. R., Handlon, A. L. & Oppenheimer, N. J. Pyridine coenzyme analogs. 3. Synthesis of three NAD+ analogs containing a 2'-deoxy-2'-substituted nicotinamide arabinofuranosyl moiety. *J. Org. Chem.* **56,** 3608–3613 (1991).
47. Sauve, A. A. & Schramm, V. L. Mechanism-based inhibitors of CD38: A mammalian cyclic ADP-ribose synthetase. *Biochemistry* **41,** 8455–8463 (2002).
48. Moreno-García, M. E. *et al.* CD38 is expressed as noncovalently associated homodimers on the surface of murine B lymphocytes. *Eur. J. Biochem.* **271,** 1025–1034 (2004).
49. Heldin, C.-H. Dimerization of cell surface receptors in signal transduction. *Cell* **80,** 213–223 (1995).
50. MacDonald, R. J. *et al.* Probing the requirement for CD38 in retinoic acid-induced HL-60 cell differentiation with a small molecule dimerizer and genetic knockout. *Sci. Rep.* **7,** 17406 (2017).
51. Förster, R. *et al.* A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* **87,** 1037–1047 (1996).

52. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* **192,** 1545–1552 (2000).
53. Schaerli, P. *et al.* CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J. Exp. Med.* **192,** 1553–1562 (2000).
54. Moser, B. CXCR5, the defining marker for follicular B helper T (T_{FH}) cells. *Front. Immunol.* **6,** 296 (2015).
55. Bénézech, C. *et al.* Inflammation-induced formation of fat-associated lymphoid clusters. *Nat. Immunol.* **16,** 819–828 (2015).
56. Legler, D. F. *et al.* B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J. Exp. Med.* **187,** 655–660 (1998).
57. Suto, H., Katakai, T., Sugai, M., Kinashi, T. & Shimizu, A. CXCL13 production by an established lymph node stromal cell line via lymphotoxin-beta receptor engagement involves the cooperation of multiple signaling pathways. *Int. Immunol.* **21,** 467–476 (2009).
58. Battle, T. E., Levine, R. A. & Yen, A. Retinoic acid-induced blr1 expression promotes ERK2 activation and cell differentiation in HL-60 cells. *Exp. Cell Res.* **254,** 287–298 (2000).
59. El-Haibi, C. P. *et al.* Differential G protein subunit expression by prostate cancer cells and their interaction with CXCR5. *Mol. Cancer* **12,** 64 (2013).
60. Barroso, R. *et al.* EBI2 regulates CXCL13-mediated responses by heterodimerization with CXCR5. *FASEB J.* **26,** 4841–4854 (2012).
61. Naor, Z., Benard, O. & Seger, R. Activation of MAPK cascades by G-protein-coupled receptors: The case of gonadotropin-releasing hormone receptor. *Trends Endocrinol. Metab.* **11,** 91–99 (2000).
62. Blaukat, A., Barac, A., Cross, M. J., Offermanns, S. & Dikic, I. G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of Galpha(q) and Galpha(i) signals. *Mol. Cell. Biol.* **20,** 6837–6848 (2000).
63. Law, N. C., White, M. F. & Hunzicker-Dunn, M. E. G protein-coupled receptors (GPCRs) that signal via protein kinase A (PKA) cross-talk at insulin receptor substrate 1 (IRS1) to activate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. *J. Biol. Chem.* **291,** 27160–27169 (2016).
64. Congleton, J., MacDonald, R. & Yen, A. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. *Leukemia* **26,** 1180–1188 (2012).
65. Brandvold, K. R., Steffey, M. E., Fox, C. C. & Soellner, M. B. Development of a highly selective c-Src kinase inhibitor. *ACS Chem. Biol.* **7,** 1393–1398 (2012).
66. Davis, M. I. *et al.* Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* **29,** 1046–1051 (2011).
67. Kantarjian, H. M. *et al.* Dasatinib or imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: 2-Year follow-up from a randomized phase 3 trial (DASISION). *Blood* **119,** 1123–1129 (2012).
68. Kantarjian, H. M. *et al.* Bosutinib safety and management of toxicity in leukemia patients with resistance or intolerance to imatinib and other tyrosine kinase

inhibitors. *Blood* **123,** 1309–1318 (2014).
69. MacDonald, R. J. *et al.* Src family kinase inhibitor bosutinib enhances retinoic acid-induced differentiation of HL-60 leukemia cells. *Leuk. Lymphoma* 1–11 (2018).
70. Shen, M. & Yen, A. c-Cbl interacts with CD38 and promotes retinoic acid-induced differentiation and G0 arrest of human myeloblastic leukemia cells. *Cancer Res.* **68,** 8761–8769 (2008).
71. Frauenstein, K. *et al.* Activation of the aryl hydrocarbon receptor by the widely used Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2). *Arch. Toxicol.* **89,** 1329–1336 (2014).
72. Scholl, S. *et al.* Additive effects of PI3-kinase and MAPK activities on NB4 cell granulocyte differentiation: Potential role of phosphatidylinositol 3-kinase γ. *J. Cancer Res. Clin. Oncol.* **134,** 861–872 (2008).
73. Nagao, T. *et al.* Proliferation and survival signaling from both Jak2-V617F and Lyn involving GSK3 and mTOR/p70S6K/4EBP1 in PVTL-1 cell line newly established from acute myeloid leukemia transformed from polycythemia vera. *PLoS One* **9,** e84746 (2014).
74. Eckschlager, T., Plch, J., Stiborova, M. & Hrabeta, J. Histone deacetylase inhibitors as anticancer drugs. *Int. J. Mol. Sci.* **18,** E1414 (2017).
75. He, L. Z. *et al.* Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. *J. Clin. Invest.* **108,** 1321–1330 (2001).
76. Amin, H. M., Saeed, S. & Alkan, S. Histone deacetylase inhibitors induce caspase-dependent apoptosis and downregulation of daxx in acute promyelocytic leukaemia with t(15;17). *Br. J. Haematol.* **115,** 287–297 (2001).
77. Kosugi, H. *et al.* In vivo effects of a histone deacetylase inhibitor, FK228, on human acute promyelocytic leukemia in NOD / Shi-scid/scid mice. *Jpn. J. Cancer Res.* **92,** 529–536 (2001).
78. Bolden, J. E., Peart, M. J. & Johnstone, R. W. Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov.* **5,** 769–784 (2006).
79. Deubzer, H. *et al.* Novel valproic acid derivatives with potent differentiation-inducing activity in myeloid leukemia cells. *Leuk. Res.* **30,** 1167–1175 (2006).
80. Bunaciu, R. P. & Yen, A. Activation of the aryl hydrocarbon receptor ahr promotes retinoic acid-induced differentiation of myeloblastic leukemia cells by restricting expression of the stem cell transcription factor Oct4. *Cancer Res.* **71,** 2371–2380 (2011).
81. Yen, A. & Varvayanis, S. DMSO, sodium butyrate, and TPA induce hypophosphorylation of RB with HL-60 cell differentiation. *In Vitro Cell. Dev. Biol. Anim.* **31,** 164–167 (1995).
82. Hu, J., Jing, H. & Lin, H. Sirtuin inhibitors as anticancer agents. *Future Med. Chem.* **6,** 945–966 (2014).
83. Jing, H. *et al.* A SIRT2-selective inhibitor promotes c-Myc oncoprotein degradation and exhibits broad anticancer activity. *Cancer Cell* **29,** 297–310 (2016).
84. Shah, A. A., Ito, A., Nakata, A. & Yoshida, M. Identification of a selective SIRT2 inhibitor and its anti-breast cancer activity. *Biol. Pharm. Bull.* **39,** 1739–1742 (2016).
85. Sunami, Y. *et al.* Inhibition of the NAD-dependent protein deacetylase SIRT2

induces granulocytic differentiation in human leukemia cells. *PLoS One* **8,** e57633 (2013).
86. Deng, A., Ning, Q., Zhou, L. & Liang, Y. SIRT2 is an unfavorable prognostic biomarker in patients with acute myeloid leukemia. *Sci. Rep.* **6,** (2016).
87. Xu, H. *et al.* SIRT2 mediates multidrug resistance in acute myelogenous leukemia cells via ERK1/2 signaling pathway. *Int. J. Oncol.* 613–623 (2015).
88. Tyner, J. W. Functional genomics for personalized cancer therapy. *Sci. Transl. Med.* **6,** (2014).
89. Jackson, S. E. & Chester, J. D. Personalised cancer medicine. *Int. J. Cancer* **137,** 262–266 (2015).

APPENDIX

ADDITIONAL PUBLICATIONS WITH CONTRIBUTIONS BY THE AUTHOR

Congleton, J., MacDonald, R. & Yen, A. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK dependent differentiation of myeloid leukemia cells. *Leukemia* **26**, 1180-1188 (2012).

Abstract

All-*trans*-retinoic-acid (ATRA)-induced differentiation of human myeloid leukemia cells is characterized by persistent MAPK signaling. Fragmentary data suggests Src family kinase (SFK) inhibitors enhance differentiation and thus have potential therapeutic value. The present study shows that SFK inhibitors PP2 and dasatinib enhance aspects of MAPK signaling and regulate a panel of differentiation markers including CD11b and $p47^{phox}$. HL-60 and NB4 myeloid leukemia cells show accelerated ATRA-induced G1/0 arrest/differentiation with inhibitor co-treatment. We also identified components of a Lyn- and c-Raf-containing MAPK signaling complex augmented by the inhibitors. PP2 and dasatinib increased ATRA-induced expression of Lyn and c-Raf (total and c-RafpS259) and their interaction. The Lyn-associated serine/threonine kinase CK2 also complexed with c-Raf and c-RafpS259, and the KSR1 scaffold protein bound c-Raf, Lyn, and ERK. c-Raf/ERK association was increased by the inhibitors, which is significant since ERK may cause c-Raf C-terminal domain (CTD) phosphorylation in a putative feedback mechanism. Consistent with this, inhibitor treatment caused more CTD phosphorylation. Lyn knockdown decreased c-Raf CTD and S259 phosphorylation. This is the first evidence suggesting SFK inhibitors enhance ATRA-induced differentiation through a possible feedback loop involving KSR1-scaffolded c-Raf and ERK complexed with Lyn and CK2.

Shrimp, J. H., Hu, J., Dong, M., Wang, B. S., MacDonald, R., Jiang, H., Hao, Q., Yen, A. & Lin, H. Revealing CD38 cellular localization using a cell permeable, mechanism-based fluorescent small-molecule probe. *J. Am. Chem. Soc.* **136,** 5656-5663 (2014).

Abstract

Nicotinamide adenine dinucleotide (NAD) is increasingly recognized as an important signaling molecule that affects numerous biological pathways. Thus, enzymes that metabolize NAD can have important biological functions. One NAD-metabolizing enzyme in mammals is CD38, a type II transmembrane protein that converts NAD primarily to adenosine diphosphate ribose (ADPR) and a small amount of cyclic adenosine diphosphate ribose (cADPR). Localization of CD38 was originally thought to be only on the plasma membrane, but later reports showed either significant or solely, intracellular CD38. With the efficient NAD-hydrolysis activity, the intracellular CD38 may lead to depletion of cellular NAD, thus producing harmful effects. Therefore, the intracellular localization of CD38 needs to be carefully validated. Here, we report the synthesis and application of a cell permeable, fluorescent small molecule (SR101-F-araNMN) that can covalently label enzymatically active CD38 with minimal perturbation of live cells. Using this fluorescent probe, we revealed that CD38 is predominately on the plasma membrane of Raji and retinoic acid (RA)-treated HL-60 cells. Additionally, the probe revealed no CD38 expression in K562 cells, which was previously reported to have solely intracellular CD38. The finding that very little intracellular CD38 exists in these cell lines suggests that the major enzymatic function of CD38 is to hydrolyze extracellular rather than intracellular NAD. The fluorescent activity-based probes that we developed allow the localization of CD38 in different cells to be determined, thus enabling a better understanding of the physiological function.

Bunaciu, R. P., Jensen, H. A.*, MacDonald, R. J.*, LaTocha, D. H., Varner, J. D. & Yen, A. 6-formylindolo(3,2-b)carbazole (FICZ) modulates the signalsome responsible for RA-induced differentiation of HL-60 myeloblastic leukemia cells. *PLoS One.* **10,** e0135668 (2015).

*These authors contributed equally to this work

Abstract

6-Formylindolo(3,2-b)carbazole (FICZ) is a photoproduct of tryptophan and an endogenous high affinity ligand for aryl hydrocarbon receptor (AhR). It was previously reported that, in patient-derived HL-60 myeloblastic leukemia cells, retinoic acid (RA)-induced differentiation is driven by a signalsome containing c-Cbl and AhR. FICZ enhances RA-induced differentiation, assessed by expression of the membrane differentiation markers CD38 and CD11b, cell cycle arrest and the functional differentiation marker, inducible oxidative metabolism. Moreover, FICZ augments the expression of a number of the members of the RA-induced signalsome, such as c-Cbl, Vav1, Slp76, PI3K, and the Src family kinases Fgr and Lyn. Pursuing the molecular signaling responsible for RA-induced differentiation, we characterized, using FRET and clustering analysis, associations of key molecules thought to drive differentiation. Here we report that, assayed by FRET, AhR interacts with c-Cbl upon FICZ plus RA-induced differentiation, whereas AhR constitutively interacts with Cbl-b. Moreover, correlation analysis based on the flow cytometric assessment of differentiation markers and western blot detection of signaling factors reveal that Cbl-b, p-p38α and pT390-GSK3β, are not correlated with other known RA-induced signaling components or with a phenotypic outcome. We note that FICZ plus RA elicited signaling responses that were not typical of RA alone, but may represent alternative differentiation-driving pathways. In clusters of signaling molecules seminal to cell differentiation, FICZ co-administered with RA

augments type and intensity of the dynamic changes induced by RA. Our data suggest relevance for FICZ in differentiation-induction therapy. The mechanism of action includes modulation of a SFK and MAPK centered signalsome and c-Cbl-AhR association.

Bunaciu, R. P., MacDonald, R. J., Gao, F., Johnson, L. M., Varner, J. D., Wang, X., Nataraj, S., Guzman, M. L. & Yen, A. Potential for subsets of wt-NPM1 primary AML blasts to respond to retinoic acid treatment. *Oncotarget* **9,** 4134-4149 (2017).

Abstract

Acute myeloid leukemia (AML) has high mortality rates, perhaps reflecting a lack of understanding of the molecular diversity in various subtypes and a lack of known actionable targets. There are currently 12 open clinical trials for AML using combination therapeutic modalities including all-*trans* retinoic acid (RA). Mutant nucleophosmin-1, proposed as a possible marker for RA response, is the criterion for recruiting patients in three active RA phase 3 clinical trials. We tested the ability of RA alone or in combination with either bosutinib (B) or 6-formylindolo(3,2-b) carbazole (F) to induce conversion of 12 *de novo* AML samples toward a more differentiated phenotype. We assessed levels of expression of cell surface markers associated with differentiation, aldehyde dehydrogenase activity, and glucose uptake activity. Colony formation capacity was reduced with the combined treatment of RA and B or F, and correlated with modulation of a c-Cbl/Lyn/c-Raf-centered signalsome. Combination treatment was in most cases more effective than RA alone. Based on their responses to the treatments, some primary leukemic samples cluster closer to HL-60 cells than to other primary samples, suggesting that they may represent a hitherto undefined AML subtype that is potentially responsive to RA in a combination differentiation therapy.

APPENDIX

ADDITIONAL FLOW CYTOMETRIC DATA

We performed single experiments to determine cell surface expression of CD32a and CD45 using antibodies obtained from Abcam (Cambridge, UK). Antibodies were conjugated with Alexa Fluor 594 following the procedure described in Chapter 3 and analyzed on a Becton Dickinson FACS Aria III SORP (Franklin Lakes, NJ) as described in Chapter 3. Briefly, HL-60 cells were treated with 1 μM RA for 48 h as indicated, stained with antibodies for 1 h at 37^{o}C, and washed with PBS prior to analysis. Channel means are shown in the table below.

Sample	CD32a Mean Expression	CD45 Mean Expression
HL-60	2747	1899
HL-60 RA	5742	3153

We also probed for interaction between CXCR5 and RARα as well as CXCR5 and RXRα in live HL-60 and CXCR5^{+} cells treated with RA for 24 h as indicated. RARα and RXRα antibodies were obtained from Abcam (Cambridge, UK) and Alexa Fluor 488-conjugated CXCR5 antibody was obtained from BioLegend (San Diego, CA). RARα and RXRα antibodies were conjugated to Alexa Fluor 546 as described in Chapter 4. Cells were stained and analyzed on a Becton Dickinson FACS Aria III SORP (Franklin Lakes, NJ) as described in Chapter 4. We did not include the data in the chapter due to uncertainty regarding the specificity of the antibodies; as this was an exploratory venture we did not include positive and negative controls for RARα and RXRα staining at the cell surface. The proteins are nuclear receptors and are generally not known to be expressed at the cell membrane, although there is one report in which RARα was detected there.[1] Our expression and FRET data are shown in Figure A1.

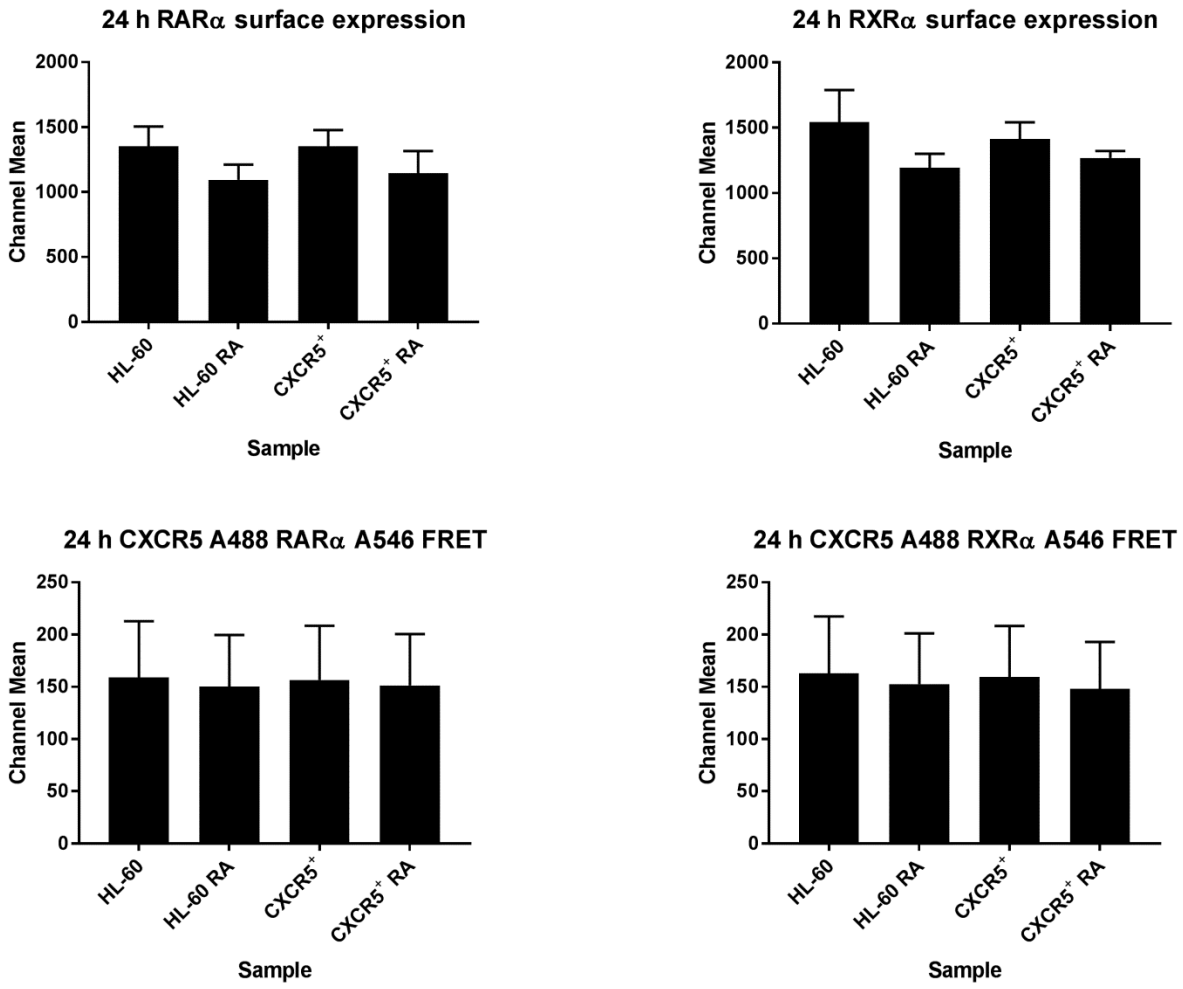


Figure A1: Surface expression of RARα and RXRα and flow cytometry FRET between CXCR5 and RARα or CXCR5 and RXRα. Primary antibodies conjugated to Alexa Fluor 488 (donor) or Alexa Fluor 546 (acceptor) were used to label CXCR5 and RARα and RXRα on the cell surface. Mean fluorescence intensity (n = 3) in the RARα and RXRα channels are shown in HL-60 and CXCR5$^+$ cells with or without 24 h RA treatment as indicated. FRET channel means (n = 3) in the FRET channels are shown in HL-60 and CXCR5$^+$ cells with or without 24 h RA treatment as indicated. Error bars indicate standard error of the mean.

REFERENCE

1. Piskunov, A. & Rochette-Egly, C. A retinoic acid receptor RARα pool present in membrane lipid rafts forms complexes with G protein αQ to activate p38MAPK. *Oncogene* **31,** 3333–3345 (2012).