

**SIGNAL REGULATION BY CD38, LYN, AND MAPK-ASSOCIATED MOLECULES  
DURING ALL-*TRANS* RETINOIC ACID-INDUCED DIFFERENTIATION OF ACUTE  
MYELOGENOUS LEUKEMIA CELLS**

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

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January 2012

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# **SIGNAL REGULATION BY CD38, LYN, AND MAPK-ASSOCIATED MOLECULES DURING ALL-TRANS RETINOIC ACID-INDUCED DIFFERENTIATION OF ACUTE MYELOGENOUS LEUKEMIA CELLS**

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Cornell University, 2012

## **Abstract**

All-*trans* retinoic acid (ATRA) is successful in treating acute promyelocytic leukemia but has shown limited success with other cancers. Identifying signaling pathways that confer ATRA responsiveness and compounds that enhance the effects of the drug could expand its therapeutic efficacy. The leukocyte antigen CD38 is an ectoenzyme and receptor that propels MAPK signaling and ATRA-induced differentiation when overexpressed. CD38 enzymatic activity regulates calcium flux and NAD metabolism while its receptor functions drive phosphorylation of ERK, c-Cbl, and p85 PI3K. We found that crippling enzymatic activity with inhibitors or a point mutation (CD38 E226Q) did not affect ATRA induction, while a cytosolic truncation (CD38  $\Delta$ 11-20) that prevented CD38 membrane expression crippled ATRA-induced differentiation. This indicated that CD38 receptor function is important for ATRA induction. In contrast, we found CD38  $\Delta$ 11-20 cells responded to the monocytic inducer 1,25-dihydroxyvitamin D3 similar to wild-type cells, suggesting CD38 signaling is important for granulocytic but not monocytic maturation. We also found that CD38 may sustain ERK phosphorylation to maintain a cellular precommitment memory associated with ATRA exposure. Since CD38 receptor functions appeared necessary for ATRA induction we identified CD38 interaction partners. These included SLP-76, Vav1, and Lyn, and we found that this complex was interrupted by inhibiting Lyn. Lyn inhibition also blocked ATRA- and CD38-stimulated

phosphorylation of c-Cbl and p85 PI3K, suggesting some aspects of CD38 signaling are dependent on Lyn kinase activity. Finally we found that the Lyn inhibitors PP2 and dasatinib enhanced ATRA-induced differentiation. Co-treatment with ATRA plus either inhibitor upregulated total Lyn and c-RafpS259, and increased associations between Lyn/c-Raf and c-Raf/ERK. This was consistent with increased c-Raf C-terminus phosphorylation that may be regulated by ERK-propelled feedback signaling within a KSR1 scaffold. PP2 and dasatinib had different effects on Lyn activity suggesting that Lyn acts as an adaptor/scaffold independent of its kinase function. These results provide valuable insight as to how ATRA-regulated molecules including CD38, Vav1, SLP-76, Lyn, and the Raf/MEK/ERK axis may enhance the differentiation of acute myelogenous leukemia cells. This report also identifies new therapeutic targets and compounds that may be used in combination with ATRA to improve its clinical efficacy.

## **BIOGRAPHICAL SKETCH**

Johanna Congleton began her education as a journalist and public communications major at Syracuse University in 1994, and received her BS in Communications in 1998. After graduating she focused on writing and program management with non-profit environmental organizations including Clean Water Action and the Sierra Club national headquarters in San Francisco. In 2001 she began work with Physicians for Social Responsibility-Los Angeles, where she served as Public Health Associate and directed a variety of environmental health initiatives. She worked extensively with physicians and other health care providers to implement education programs for clinicians, patients, and the general public on human exposure to environmental chemicals. This work inspired her interest in environmental health sciences and toxicology, and in May 2004 she moved to New Orleans to pursue an MSPH degree at Tulane University's School of Public Health and Tropical Medicine (SPHTM) in the Department of Environmental Health Sciences. She also founded and served as Executive Director of Physicians for Social Responsibility-Louisiana, which provided health assistance services to people impacted by Hurricanes Katrina and Rita. In the fall of 2006, she enrolled at Cornell University in the field of Environmental Toxicology, studying with Dr. Andrew Yen to earn a Ph.D. She plans to continue to use her experience and education in support of programs that advance public health and environmental science education, research, and policies.

Dedicated to my mother Nancy, my father William, my sister Christina, and my husband

Stephen.

## ACKNOWLEDGMENTS

I give my deepest thanks to Dr. Andrew Yen, my Principle Investigator and Committee Chair, who has extended his encouragement, support, and guidance throughout my graduate studies at Cornell University. His thoughtfulness and experience have been integral to my success.

I would also like to thank my Ph.D. committee members, Dr. Hening Lin and Dr. Holger Sondermann. They have given me valuable insight and advice that has supported and shaped my research. I also thank the members of Dr. Lin's lab who have collaborated with me on several studies, in particular Dr. Hong Jiang.

I am grateful to my lab members who have given me advice, support, and collaborative opportunities that have been invaluable to my studies at Cornell. I thank Dr. Gudren Reiterer, Dr. Rodica Petruta Bunaciu, Dr. Miaoqing Shen, Dr. Wendy Mercer, Robert MacDonald, and Holly Jensen. I also thank Dr. Jeffery Varner, Satya Nayakpradesh, and Ryan Tasseff from the Department of Chemical Engineering in their collaboration on several research projects and manuscripts. I thank Dr. James Smith and Lavanya Gowri Sayam at the Cornell Biomedical Science Flow Cytometry Core Lab for their assistance with FACS sorting and flow cytometric analysis. I also thank Dr. Stephen Bloom for serving as my field-appointed committee member, and Cindy Uhrovic for providing a great amount of administrative assistance and personal support.

I would also like to acknowledge the financial support I have received from the National Institutes of Health (NIH), the National Institute of Environmental Health Sciences (NIEHS), and New York State Stem Cell Science (NYSTEM).

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## LIST OF ABBREVIATIONS

AML	Acute myeloblastic leukemia
APL	Acute promyelocytic leukemia
ADPR	Adenosine diphosphate ribose
ALL	Acute Lymphocytic Leukemia
ATRA	All- <i>trans</i> retinoic acid
cADPR	Cyclic adenosine diphosphate ribose
c-FMS	Colony-stimulating factor-1 receptor
Ca <sup>2+</sup>	Calcium
c-Cbl	Casitas B-lineage Lymphoma
CD	Cluster of differentiation
CK2	Casein kinase 2
CLL	Chronic myelogenous leukemia
CSF	Colony stimulating factor
D3	1,25-dihydroxyvitamin D3
ERK	Extracellular Regulated MAP Kinase
Fgr	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
FRET	Fluorescence resonance energy transfer
GEF	Guanine nucleotide exchange factor
Ig	Immunoglobulin
INF	Interferon
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based <u>i</u> nhibitory motifs
KSR1	Kinase suppressor of ras 1
Lck	Lymphocyte-specific protein tyrosine kinase
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
m(Ab)	monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
NK	Natural killer cells

NAM	Nicotinamide
NAADP+	Nicotinamide acid adenine dinucleotide phosphate
NAD+	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PLC	Phospholipase C
PI3K	Phosphoinositide Kinase-3
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RB	Retinoblastoma
RXR	Retinoid X receptor
siRNA	Silent interfering RNA
shRNA	Small hairpin RNA
SLP-76	SH2 domain containing leukocyte protein of 76kDa
Syk	Spleen tyrosine kinase
SFK	Src family kinase
SH2	Src homology 2

## CHAPTER I: INTRODUCTION

### 1.1 All-*trans* retinoic acid in the treatment of leukemia

All-*trans* retinoic acid (ATRA) has proven highly effective in treating patients with acute promyelocytic leukemia (APL) which bears the characteristic t(15,17) chromosomal translocation. However, the therapeutic use of ATRA has significant limitations [1-5]. First, leukemias that are t(15,17) negative typically do not respond well to ATRA treatment, and retinoic acid has shown restricted efficacy in treating other types of cancers. Therefore the success of ATRA as a therapeutic option has been fairly limited. In addition, some patients develop resistance to ATRA or experience post-treatment relapse (which may also be characterized by resistance). Therefore it is important to identify cell signaling pathways that confer ATRA responsiveness to understand the mechanisms of its therapeutic value. This may assist in the identification of molecules and signaling cascades that could constitute new therapeutic targets, leading to the improvement of ATRA's efficacy and broadening its usefulness in other types of cancers. It is also of significant interest to identify new compounds that can be used in combination with ATRA to improve clinical outcomes. For example, the discovery of molecules that, in combination with ATRA, accelerate cell cycle arrest and differentiation could be particularly useful in treating patients who have relapsed or developed resistance.

### 1.2 The t(15,17) negative HL-60 human acute myeloblastic leukemia cell line is a model to study ATRA responsiveness

Bipotent human acute myelogenous leukemia (AML) HL-60 cells do not bear the t(15,17) translocation, yet they are still ATRA-responsive. It is a model cell line for studying induced

differentiation, since treatment with either ATRA or 1,25-dihydroxyvitamin D3 (D3) results in granulocytic or monocytic maturation, respectively. Treatment of HL-60 cells with retinoic acid leads to terminal differentiation evidenced by the upregulated expression of classical differentiation markers including CD38 and CD11b, inducible oxidative metabolism, and G1/0 cell cycle arrest. The mechanisms by which ATRA drives t(15,17) negative HL-60 cells to differentiate remain unclear. Since ATRA has shown little clinical efficacy in AML treatment, it is important to identify the proteins and signaling cascades that confer ATRA responsiveness to non-APL HL-60 cells. The investigation of molecular targets and drugs that modulate or enhance ATRA induction may have a significant impact on the therapeutic potential of retinoic acid in other types of cancers.

### **1.3 The role of the leukocyte antigen CD38 in leukemia is enigmatic**

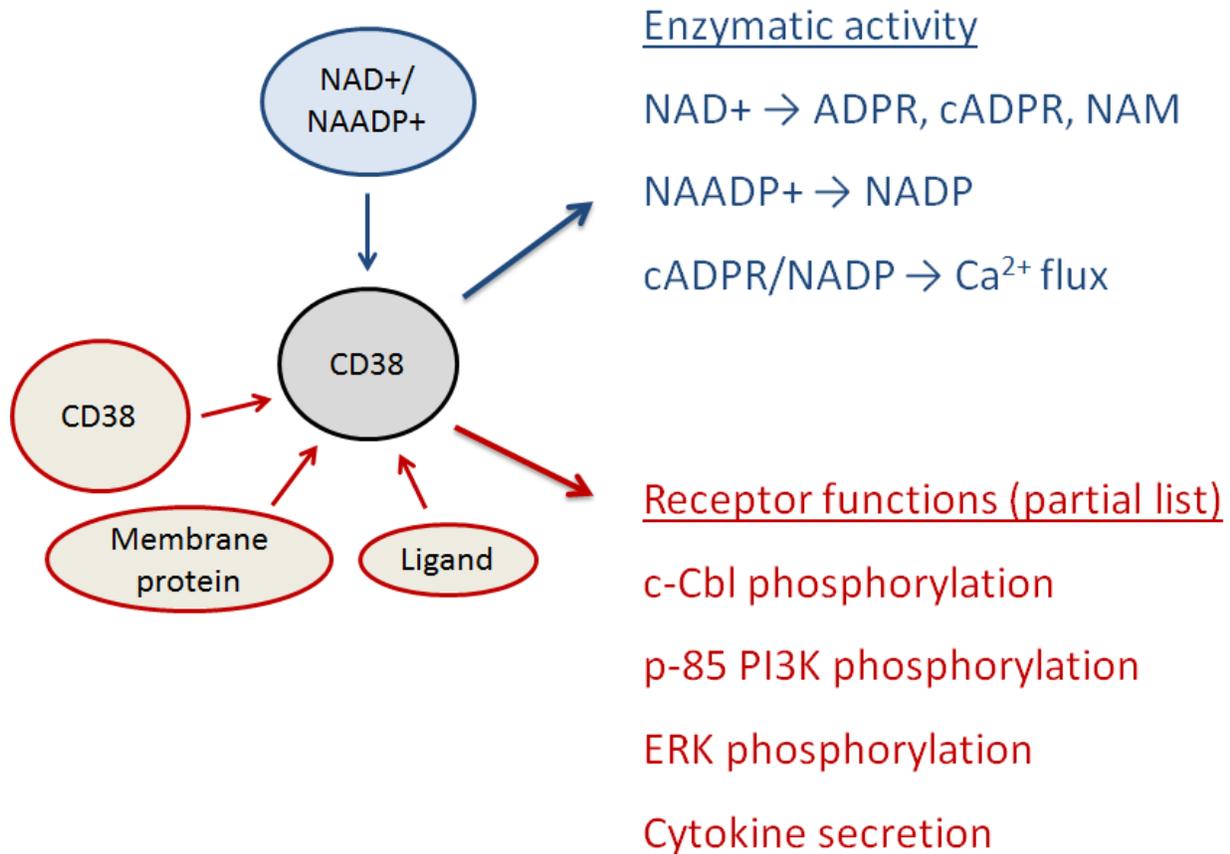
The leukocyte antigen CD38 is a 45kD type II transmembrane receptor and ectoenzyme that is differentially expressed in a variety of cells. It has been negatively and positively implicated in the prognosis of several diseases, including chronic lymphocytic leukemia (CLL), acquired immune deficiency syndrome (AIDS), and diabetes (reviewed in [6]). CD38 is an early biomarker of ATRA-induced differentiation in the HL-60 cell line yet its role in this process is unclear. CD38 contains a retinoic acid response element (RARE) in the first intron and transcription is regulated by retinoic acid receptor- $\alpha$  (RAR- $\alpha$ ) [7, 8]. The antigen is detectable after 6 hours of treatment and reaches maximum expression within 16 hours [9]. A stable transfectant cell line that overexpresses wild-type CD38 shows an enhanced rate of differentiation indicated by increased inducible oxidative metabolism by 48 hours and G1/0 arrest by 72 hours [9]. CD38 overexpression and agonist interaction also drives MAPK signaling through the c-Raf/MEK/ERK axis, which is required for induced differentiation [10-15]. In

addition, RNAi directed toward CD38 cripples ATRA induction [16]. These reports suggest that ATRA-induced myeloid differentiation in HL-60 cells may depend in part on the early expression of CD38 and the signaling cascades it regulates. Other studies support a role for CD38 in white blood cell differentiation and growth suppression, particularly B lymphocytes [17-19].

In contrast, CD38 is considered a negative prognostic indicator for CLL and may promote survival and proliferation through ERK1/2 signaling [20]. Additional reports show that CD38 ligand-induced signaling results in the proliferation of myeloid leukemia patient cells and cell lines [21, 22] or that agonist ligation prevents apoptosis [23]. Also, CD38 expression in HeLa cells shortens their doubling time [24]. Therefore the role of CD38 in leukemia remains enigmatic. The diverse cellular outcomes modulated by the antigen may reflect the function of different domains in different contexts. Identification of the domains that regulate ATRA induction would be important in elucidating how CD38 functions in a differentiating leukemia cell.

#### **1.4 Receptor and enzymatic functions of CD38**

CD38 has both enzymatic and receptor functions (Figure 1.1). As an ectoenzyme, it catalyzes the formation of adenosine diphosphate ribose (ADPR), cyclic ADPR (cADPR), and nicotinamide (NAM) from nicotinamide adenine dinucleotide (NAD<sup>+</sup>) under neutral pH; or nicotinic acid adenine dinucleotide phosphate (NAADP<sup>+</sup>) from nicotinic adenine dinucleotide phosphate (NADP) under acidic conditions [25]. The E226 residue of CD38 is considered essential for catalytic activity, since mutagenesis of this site results in a loss of enzymatic function [26-28]. Both cADPR and NAADP<sup>+</sup> facilitate calcium signaling. CD38 cADPR catalysis mediates



**Figure 1.1 Enzymatic and receptor functions of CD38.** CD38 extracellular enzymatic activity (blue) metabolizes NAD<sup>+</sup> to ADPR, cADPR, and NAM, and NAADP<sup>+</sup> to NADP. cADPR and NADP are second messengers that modulate calcium flux. Receptor functions (red) are regulated by extracellular stimulus from ligands, other membrane proteins, or CD38 dimer/oligomerization. In HL-60 and NB4 cells, CD38 signaling transduction results in phosphorylation of c-Cbl, p-85 PI3K, and ERK. CD38 also regulates a variety of signaling mechanisms in other hematopoietic cells, including cytokine secretion.

calcium mobilization from the endoplasmic reticulum in astrocytes, cardiomyocytes, and airway smooth muscle cells, and is important for chemotactic responses in neutrophils [29-31].

The role of CD38 enzymatic activity in the context of ATRA-induced differentiation is not understood. ATRA-treated HL-60 cells release nuclear calcium in response to cADPR production which correlates with the presence of nuclear CD38 protein, suggesting a role in differentiation [32]. Another report shows that the CD38/cADPR/Ca<sup>2+</sup> signaling axis promotes proliferation and delays differentiation in PC12 cells [33]. ATRA treatment causes a decrease in total cellular calcium levels, and experiments inhibiting calcium flux also suggest independence [34, 35]. Investigation of CD38 enzymatic activity after ATRA treatment would elucidate if this function is necessary for differentiation.

CD38 also has receptor functions that participate in diverse signaling mechanisms that vary with cell type and differentiation status [36]. Membrane-expressed CD38 forms lateral associations with a variety of surface receptors to facilitate signaling. These include CD3 on T lymphocytes; surface Ig, CD19, and CD21 on B cells; and CD16 on NK cells [37-39]. These lateral membrane-localized interactions result in cytosolic protein complex assembly, which include CD38 association with PI3K and Lyn [40, 41]. CD38 m(Ab) agonist ligation (which can facilitate dimerization) results in the phosphorylation of c-Cbl and ERK and may also coordinate protein complex assembly [13-15, 19, 42]. Therefore, the receptor functions of CD38 can depend on interaction with itself and a variety of membrane- or cytosol-localized proteins.

The receptor functions of CD38 may be independent of enzymatic activity. For example, CD38-mediated signaling is dependent on conformational changes of the ectodomain that result in downstream tyrosine kinase activity, and these functions are enzyme-independent [43, 44]. Since

CD38 enzymatic function and receptor signaling can drive diverse cellular outcomes, and since these two functions can operate independently, there is interest in discovering which molecules participate in the regulation of CD38-facilitated signaling cascades. Identification of such proteins could provide insight regarding how CD38 propels myeloid differentiation, and distinguish additional proteins and effectors that could be key regulators of signaling pathways required for ATRA induction.

### **1.5 Known and potential CD38-associated molecules including c-Cbl, p85 PI3K, Vav1, and SLP-76 regulate signaling and differentiation**

Proteins that show association or potential interaction with CD38, or that are involved in CD38-stimulated signaling, also show ATRA-related regulatory roles. One such protein is the E3 ubiquitin ligase and adaptor c-Cbl which, like CD38, promotes MAPK signaling and ATRA-induced differentiation when overexpressed [42, 45, 46]. CD38 and c-Cbl exist in a complex, and a c-Cbl tyrosine kinase binding (TKB) domain mutant (G306E) that does not bind CD38 also fails to drive MAPK signaling and differentiation [45]. This suggests that CD38-driven c-Cbl phosphorylation and/or its interaction with CD38 could cooperatively propel myeloid differentiation by forming critical signaling complexes at the cell membrane. IRF-1 also binds c-Cbl and drives ATRA induction, as well as MAPK signaling and CD38 expression [48] indicating another potential CD38 partner involved in propelling the effects of ATRA.

c-Cbl is also known to interact with Vav1, SLP-76, and the p85 regulatory subunit of PI3K, which suggests these proteins also interact with CD38 [45, 46, 49, 50]. c-Cbl, SLP-76, and Vav1 protein expression and p85 PI3K activity are upregulated during myeloid differentiation, suggesting they have a functional role [51-55]. These proteins also form complexes in myeloid cells during ATRA induction which may participate in CD38-coordinated signaling events.

For example, CD38 stimulation by the agonists IB4 and T16 induces phosphorylation of the p85 regulatory subunit of PI3K and its association with c-Cbl, which is correlated with normal and leukemic B cell growth suppression [19, 56]. Consistent with this, PI3K inhibitors relieve CD38-mediated growth suppression [19]. This suggests a negative regulatory role for a CD38/PI3K/c-Cbl complex in cell growth. Accordingly c-Cbl is reported to be a negative regulator of PI3K either through its E3 ubiquitin ligase activity [57, 58] or through sequestration. For example, c-Cbl phosphorylation at Y731 by a Src family kinase (SFKs) creates a docking site for the p85 subunit of PI3K which can negatively regulate survival [49, 59, 60]. Likewise Cbl-b promotes apoptosis in rat leukemia cells by suppressing PI3K activation while enhancing MEK/ERK activation [61]. PI3K could be important for the efficacy of ATRA in other types of cancer, since retinoic acid has shown promise in treating reproductive leiomyomas by modulating proteins involved in the PI3K cascade [62].

c-Cbl interaction with the adaptor protein SLP-76 and the guanine nucleotide exchange factor (GEF) Vav1 increases after ATRA treatment in HL-60 cells [45], and therefore Vav1 interacts with c-Cbl and SLP-76 in a differentiation-dependent manner. Vav1/c-Cbl complexes appear in the cytosol while Vav1/SLP-76 interactions occur in nuclei, suggesting that these complexes participate in localized cell signaling after ATRA treatment [63]. SLP-76 interacts with Vav1 through an SH2 binding domain, where Vav1 may serve as a scaffold to stabilize SLP-76 signaling microclusters [64, 65]. Co-expression of SLP-76 with CSF-1/c-FMS enhances ATRA-induced ERK activation, the expression of the differentiation marker paxillin, inducible oxidative metabolism, RA-induced RB tumor suppressor protein hypophosphorylation, and G1/0 cell cycle arrest [53]. Therefore, SLP-76 interaction with proteins such as c-Cbl, c-FMS, Vav1, and potentially CD38 may regulate aspects of ATRA induction including ERK signaling.

Like c-Cbl, tyrosine-phosphorylated Vav1 associates with PI3K and may facilitate the characteristic nucleoskeleton remodeling that occurs after ATRA treatment in HL-60 cells, which is independent of its activity as a GEF [63, 66]. Consistent with this, downmodulation of Vav1 in ATRA-treated HL-60 and NB4 cells impedes myeloid differentiation, nucleoskeleton remodeling, and affects differentiation-related protein expression [55].

Phosphorylation of Vav1 at Y745 is believed to play a critical role in ATRA-induced maturation of leukemic NB4 cells since mutation of this residue interferes with retinoic acid-propelled CD11b  $\alpha$ -integrin receptor expression and cell migration [67]. Vav1 regulates the ATRA-induced expression of CD11b by recruitment to its promoter with the PU.1 transcription factor [68]. Consistent with this, Vav1-targeted siRNA resulted in significantly decreased ATRA-induced CD11b and modulated the expression of proteins that are associated with cell cycle, apoptosis, and cytoskeleton organization [55]. Overexpression of CD38 also enhances CD11b suggesting cooperation with Vav1.

Finally, CD38 drives transient MAPK signaling after agonist ligation [13-15]. In contrast, wild-type CD38 overexpression in a stable transfectant cell line creates a persistent p-ERK signal [9]. Transient or protracted signaling from this cascade can lead to either cell proliferation or differentiation respectively [69], and sustained MAPK signaling is required for ATRA-induced differentiation since inhibitors of c-Raf and MEK interrupt this process [10, 12]. Therefore, CD38 could differentially regulate MAPK signaling and perturb the magnitude and duration of the signal. This could explain the ambiguity of CD38 as a receptor that drives proliferation or growth suppression, and supports its ability to regulate diverse cellular outcomes through signal switching. For example ATRA-induced PI3K signaling, which may be regulated by CD38, can be biphasic evidenced by an initial increase in p-Akt followed by decreased Akt phosphorylation.

In this case PI3K cooperates with MAPK regulators to propel differentiation [52, 70, 71]. Since CD38 also appears capable of signal switching it could regulate the biphasic responses of effectors including PI3K and MAPK cascades.

c-Raf regulation and its post-translational modification are of particular interest in MAPK signaling induced by ATRA and driven by CD38. Expression of activated c-Raf drives ATRA-induced differentiation [72] and supports its role in propelling persistent MAPK signaling required for ATRA induction. In addition, c-Raf phosphorylated at S621 migrates to the nucleus and is associated with differentiation [73]. Finally c-Raf may be involved in feedback signaling that fine-tunes the character of MAPK signaling propelled by ATRA. For example, ERK can directly mediate feedback phosphorylation of c-Raf on residues S287, S296, and S301, which controls Raf activation and attenuates mitogenic signaling [74, 75].

### **1.6 ATRA-induced SFKs including Lyn participate in CD38-driven signaling and associate with CD38 accessory molecules**

SFKs are a unique group of enzymes that have diverse roles in cell proliferation, survival, differentiation, adhesion, and migration. These proteins regulate hematopoiesis and can also contribute to hematopoietic cancers. One historically prominent paradigm of Src action is positive regulation of MAPK signaling and cell proliferation that contributes to cell transformation (reviewed in [76]). Yet a persistent MAPK signal characterizes ATRA-induced differentiation, thus SFKs may positively regulate ATRA induction. In support of this, SFKs are known to be upregulated after ATRA treatment [77, 78].

Lyn is an SFK of particular interest due to its contrasting roles in hematopoietic cell proliferation and differentiation. On one hand, Lyn is considered a negative regulator of hematopoiesis. A

gain-of-function Y527F mutation did not result in murine hematopoietic malignancy, but Lyn <sup>-/-</sup> mice showed myeloid progenitor proliferation and tumors [79]. This and other studies also report that Lyn is required for ITIM-dependent inhibitory signaling and phosphatase activity, loss of which may lead to tumorigenesis [80]. Lyn is an important regulator of the G-CSF receptor and negatively modulates G-CSF-stimulated granulopoiesis and growth of myeloid and multipotential progenitors in the bone marrow, and proliferation in other hematopoietic cells [81-83].

SFKs including Lyn also interact with CD38-associated molecules. As previously noted, SFKs can phosphorylate c-Cbl to create a docking site for the p85 subunit of PI3K [49]. SFKs may also directly phosphorylate p85 PI3K [84]. In this report the authors suggest that c-Cbl may act as a scaffold between Src and PI3K. Lyn has specifically been linked to CD38-stimulated signaling events that result in both proliferation and differentiation. For example, CD38 stimulation of B lymphocytes obtained from Lyn-deficient mice showed defective maturation, and drugs interfering with PI3K or ERK also decreased CD38-driven differentiation [17]. 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 [85]. Likewise, in lymphoblastoid B cell membrane rafts, CD38 is associated with Lyn and promotes cell signaling [41].

### **1.7 SFK/Lyn expression and activity may regulate leukemia cell differentiation**

Reports that SFKs, particularly Lyn, can interact with CD38 and potentially modulate its receptor function motivates interest in the role of SFK/Lyn expression and activity during ATRA-induced differentiation, and if SFK inhibitors can enhance the effects of ATRA. For

example, Lyn is the predominant active SFK expressed in AML cells [86, 87], and blocking activity has been effective in slowing leukemic cell growth [88].

Several reports provide fragmentary data indicating that SFK activity and expression could also modulate ATRA differentiation induction therapy. Miranda et al. recently reported that the inhibitor PP2 potentiated ATRA-induced gene expression and enhanced the differentiation marker CD11b in myeloid NB4, HL-60, and primary APL cells [89]. Kropf et al. recently reported that dasatinib also increased ATRA-induced CD11b expression [87]. Other studies show that Lyn and Fgr are upregulated in HL-60 and NB4 myeloid leukemia cells after ATRA treatment and prevent apoptosis during granulocytic differentiation [78, 90].

SFK inhibitors are capable of both positive and negative regulatory effects on MAPK pathway components, supporting the notion that they may regulate ATRA induction. PP2 enhances Ras-independent Raf-1 activation that is mediated by Raf S621 phosphorylation [91], suggesting that SFK inhibitors are able to positively regulate Raf. This implies that SFKs could modulate c-Raf phosphorylation status and its ability to orchestrate MAPK feedback signaling. In contrast, the inhibitor dasatinib blocks MAPK activity in the absence of growth factors (GFs) and attenuates signaling in the presence of GFs in CML progenitors [92]. MAPK signal perturbation by SFKs may have implications for ATRA induction therapy, since retinoic acid results in sustained MAPK signaling which is required for HL-60 maturation [10, 12, 46].

The ability of SFKs to potentially regulate ATRA-induced differentiation and MAPK signaling is thus not understood. This motivates interest in how SFK inhibitors can affect the extent of ATRA-induced phenotypic conversion and if MAPK regulatory molecules are involved.

## **1.8 Project Overview**

The data described above motivated several principal research questions. First, it would be important to elucidate which domains of CD38 drive differentiation. Therefore we investigated whether its functions as a receptor, ectoenzyme, or both were important for ATRA induction. Second, the identification of proteins that partner with CD38 to drive signaling, and how they may regulate such activity, is integral to understanding how CD38 modulates differentiation and may point to new therapeutic targets. This led to an analysis of which proteins complexed with CD38 and how these interactions were affected by CD38 stimulation, ATRA induction, or CD38 expression in the absence of ATRA. Finally, we investigated whether compounds or drugs that impinge on CD38-associated molecules are able to enhance the efficacy of ATRA. Ultimately, these results could have substantial implications for the use of ATRA in a wider variety of leukemia types, and provide co-treatment options for patients that have developed resistance or relapsed. The results discussed in this dissertation address these research questions and provide information that could significantly impact the clinical use of ATRA.

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## CHAPTER II

### **ATRA-INDUCED HL-60 MYELOID LEUKEMIA CELL DIFFERENTIATION DEPENDS ON THE CD38 CYTOSOLIC TAIL NEEDED FOR MEMBRANE LOCALIZATION, BUT CD38 ENZYMATIC ACTIVITY IS UNNECESSARY**

#### **2.1 Abstract**

Leukocyte antigen CD38 expression is an early marker of all-*trans* retinoic acid (ATRA) stimulated differentiation in the leukemic cell line HL-60. It promotes induced myeloid maturation when overexpressed, whereas knocking it down is inhibitory. It is a type II membrane protein with an extracellular C-terminal enzymatic domain with NADase/NADPase and ADPR cyclase activity and a short cytoplasmic N-terminal tail. Here we determined whether CD38 enzymatic activity or the cytoplasmic tail is required for ATRA-induced differentiation. Neither a specific CD38 ectoenzyme inhibitor nor a point mutation that cripples enzymatic activity (CD38 E226Q) diminishes ATRA-induced differentiation or G1/0 arrest. In contrast a cytosolic deletion mutation (CD38  $\Delta$ 11-20) prevents membrane expression and inhibits differentiation and G1/0 arrest. These results may be consistent with disrupting the function of critical molecules necessary for membrane-expressed CD38 signal transduction. One candidate molecule is the Src family kinase Fgr, which failed to undergo ATRA-induced upregulation in CD38  $\Delta$ 11-20 expressing cells. Another is Vav1, which also showed only basal expression after ATRA treatment in CD38  $\Delta$ 11-20 expressing cells. Therefore, the ability of CD38 to propel ATRA-induced myeloid differentiation and G1/0 arrest is unimpaired by loss of its ectoenzyme activity. However a cytosolic tail deletion mutation disrupted membrane localization and inhibited differentiation. ATRA-induced differentiation thus does not require the CD38 ectoenzyme function, but is dependent on a membrane receptor function.

## 2.2 Introduction

All-*trans* retinoic acid (ATRA) leads to the myeloid differentiation and G1/0 arrest of HL-60 human myeloblastic leukemia cells. The process may depend on the early ATRA-induced expression of the leukocyte antigen CD38, a 45kD type II transmembrane glycoprotein that has both enzymatic and receptor functions. It is an early biomarker of ATRA-induced differentiation in the HL-60 cell line that is detectable after 6 hours of treatment and reaches maximum expression within 16 hours [1]. CD38 may play a causal role in HL-60 myeloid differentiation, since RNAi directed toward CD38 crippled ATRA induction [2]. Transfectants that overexpress wild-type CD38 show an enhanced rate of differentiation indicated by increased inducible oxidative metabolism by 48 hours and G1/0 arrest by 72 hours [1].

CD38 is an ectoenzyme that catalyzes the formation of adenosine diphosphate ribose (ADPR), cyclic ADPR (cADPR), and nicotinamide from NAD<sup>+</sup> under neutral pH; or NAADP<sup>+</sup> from NADP under acidic conditions [3]. Both cADPR and NAADP<sup>+</sup> facilitate calcium signaling. ATRA-treated HL-60 cells release nuclear calcium in response to cADPR production that correlates with the presence of nuclear CD38 protein, suggesting a role in differentiation [4]. However, ATRA-induced differentiation causes a decrease in total cellular calcium levels, and studies of calcium flux inhibition during ATRA treatment also suggested independence [5, 6]. Thus the precise role of calcium flux and its stimulation is not fully understood.

In addition to its enzymatic activity, CD38 has receptor functions that participate in diverse signaling mechanisms that vary with cell type and differentiation status [7]. Membrane-expressed CD38 forms lateral associations with CD3 on T lymphocytes; with surface Ig, CD19, and CD21 on B cells; and with CD16 on NK cells to produce signaling events [8-10]. In human

B cell precursors, ligation results in tyrosine phosphorylation of proteins such as Syk, phospholipase C- $\gamma$ , and the p85 subunit of PI3K [11]. In myeloid cells, CD38 m(Ab)-induced tyrosine phosphorylation can be mediated through Fc $\gamma$ II receptors [12]. In HL-60 cells CD38-agonist interaction also results in phosphorylation of c-Cbl, a cytosolic adapter molecule known to promote MAPK signaling and ATRA-induced differentiation [13, 14]. Fluorescence resonance energy transfer (FRET) data and immunoprecipitation experiments show that these proteins exist in a complex [15].

CD38 also drives MAPK activation after agonist ligation, which is orchestrated by Raf, MEK, and ERK [16, 17]. Transient or protracted signaling from this cascade can lead to either cell proliferation or differentiation respectively [18], and sustained MAPK signaling is required for ATRA-induced differentiation [19, 20].

In myeloid cells, CD38 signaling may promote either cell proliferation or growth inhibitory signals [21, 22]. The apparently divergent functions, particularly within myeloid cell lines, make the role of CD38 somewhat enigmatic. It may reflect the function of different domains and their relative activities in different contexts. Given that the enzymatic activity, receptor signaling, and downstream effectors of CD38 might produce divergent outcomes, and that CD38 likely participates directly in differentiation, we investigated which domains of CD38 are required for ATRA-induced HL-60 myeloid differentiation. Our results showed that the enzymatic activity of CD38 is expendable, while the transmembrane proximal cytosolic region needed for membrane expression is required.

## 2.3 Materials and Methods

*Cell culture.* HL-60 human myeloblastic leukemia cells and stable transfectant cell lines (CD38 E226Q, WT38, and CD38  $\Delta$ 11-20) were grown in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum purchased from Invitrogen (Carlsbad, CA) in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. All-*trans*-retinoic acid (ATRA) was purchased from Sigma (St. Louis, MO) and solubilized in ethanol. Cells were cultured in a final concentration of 1  $\mu$ M. Arabinosyl 2'-fluoro-2'-deoxy NAD (F-araNAD) small molecules were suspended in water and cells were cultured in a final concentration of 5  $\mu$ M. For some experiments additional 5  $\mu$ M doses were added every 24 hours to compensate for possible inhibitor degradation.

*Antibodies and reagents.* F-araNAD was synthesized following published procedures [23]. Antibodies for cytometric analysis of CD38 and CD11b, and CD38 Western blot antibody were purchased from Invitrogen and BD Pharmingen (San Jose, CA), respectively. Fgr antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); HRP anti-mouse, HRP anti-rabbit, Vav1, GAPDH, and p-ERK were purchased from Cell Signaling (Danvers, MA); and anti-total ERK from Zymed (San Francisco, CA). M-PER Mammalian Protein Extraction Reagent lysis buffer was purchased from Pierce (Rockford, IL). Protease and phosphatase inhibitors, nicotinamide guanine dinucleotide (NGD+), propidium iodide, dimethyl sulfoxide (DMSO), and 12-O-Tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma, and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCFDA) or DCFH was purchased from Invitrogen.

*Generation of stable transfectants.* WT38 cell lines were created as previously described [1]. Stable transfectants were sorted to enrich the population for cells with transfected expression by

flow cytometry using anti-CD38. CD38 E226Q was created by transfecting HL-60 cells with a pIRES-hr GFP II vector purchased from Stratagene (La Jolla, CA) containing a CD38 PCR product flanked by 5' EcoRV (GATATCATGGCCAACTGCGAGTTCAGCCCGGTG) and 3' NotI restriction sites (GCGGCCGCTCAGATGTGCAAGATGAATCCTCAGGATTTTTCAC). Site-directed mutagenesis was used to create the CD38 E226Q point mutation using the (GCACTTTTGGGAGTGTGCAAGTCCATAATTTGCAACCAG) sequence. Stable transfectants were sorted by flow cytometry based on CD38 immunostaining.

The CD38  $\Delta$ 11-20 cell line was created using an identical pIRES-hr GFP II vector with CD38 cDNA containing a deletion of amino acids 11-20 with a HindIII site bridging nucleotides 30-61. The cDNA fragment was cloned into the vector with 5' EcoRV and 3' NotI restriction sites. Segment 1: 5' EcoRV forward (GATATCAGTGAAACAGAAGGGGAGGTGCAGTTTCAG) and 3' HindIII reverse (AAGCTTCCCGGACACCGGGCTGAACTCGCAGTT); Segment 2: 3' HindIII forward (AAGCTTGCCCAACTCTGTCTTGGCGTCAGTATCCTG) and 5' NotI reverse (GCGGCCGCTCAGATGTGCAAGATGAATCCTCAGGATTT). Stable transfectants were created by flow cytometric sorting for GFP-positive cells. Stably transfected cell lines containing CD38 E226Q mutations and the CD38  $\Delta$ 11-20 truncation were further verified by mRNA isolation followed by reverse transcriptase PCR amplification and gel analysis and sequencing.

*mRNA isolation and sequencing.* Total RNA was extracted from the CD38 E226Q and CD38  $\Delta$ 11-20 cell lines with Invitrogen Trizol reagent per the manufacturer's protocol. Total RNA was measured by a spectrofluorometer (Beckman Coulter, CA), and reverse transcriptase polymerase chain reaction (RT-PCR) was performed using Invitrogen SuperScript One-Step RT-PCR with

Platinum Taq. The appropriate DNA sequences were then verified by sequencing at Cornell University's Life Sciences Core Laboratory.

*Flow cytometric phenotypic analysis.* Immunostaining for CD11b and CD38 was performed as previously described using a Becton Dickinson LSR II flow cytometer (San Jose, CA) [15].

Gating was set to exclude 95% of the negative control. Respiratory burst capability was measured as previously described [6]. Propidium iodide (PI) cell cycle analysis was performed as previously described [15].

*Western blot analysis.* For Western blot analysis, cells were pelleted, washed, and lysed with ice cold Pierce M-PER Mammalian Protein Extraction Reagent (Rockford, IL) with a protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin (Sigma); and a phosphatase inhibitor cocktail containing sodium vanadate, sodium molybdate, sodium tartrate, and imidazoleprotease (Sigma). Samples were incubated on ice and debris was pelleted. Protein content was determined by Pierce BCA protein reagent assay. Lysate was subjected to standard SDS-PAGE. Membranes were then probed with antibodies described above. All blot films shown were transferred to the same membrane and are the same exposure.

*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 Fluoro Max3, NJ) as previously described [24, 25]. For the CD38 F-araNAD inhibitor assay, cells were cultured for 48 hours untreated or in the presence of 1  $\mu$ M ATRA to induce CD38 expression. Appropriate samples were treated with 5  $\mu$ M F-araNAD or left untreated for 30 minutes with constant rotation at 37°C with protease and phosphatase

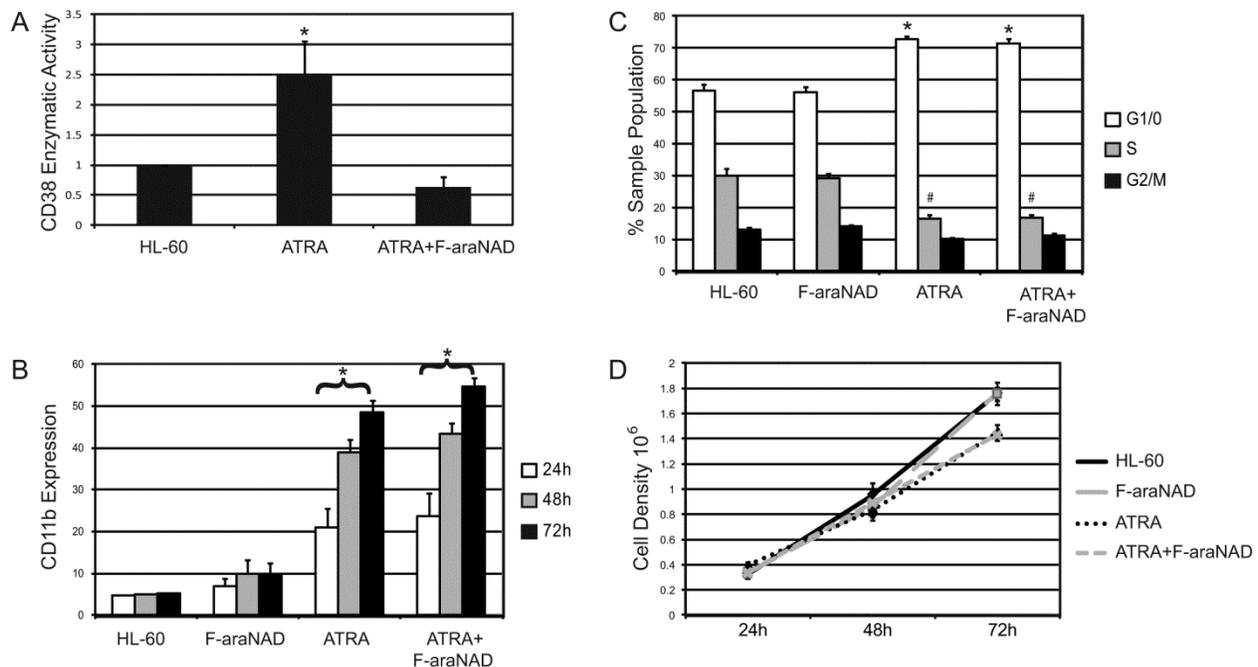
inhibitors. All samples evaluated for CD38 enzymatic activity were treated with 100  $\mu$ M NGD+ for one hour with constant rotation at 37°C. Cells were then pelleted, and supernatant was subjected to fluorometric analysis for cGDPR.

*Statistics.* Statistics were analyzed by JMP Statistical Discovery Software and Microsoft Excel statistical software.

## **2.4 Results**

*2.4.1 CD38 enzymatic inhibitors do not compromise ATRA-induced differentiation or G1/0 arrest.* CD38 enzymatic ADPR cyclase activity is important for calcium mobilization and therefore potentially relevant to differentiation. The enzymatic activity and the receptor signaling of CD38 are reportedly uncoupled, and ADPR or cADPR generation is thought to be independent of signaling mechanisms [26, 27]. Therefore, we investigated whether crippling the enzymatic activity of CD38 influenced ATRA-induced differentiation.

We first tested whether a small molecule inhibitor of CD38 can interfere with ATRA-induced HL-60 cell differentiation. Arabinosyl 2'-fluoro-2'-deoxy NAD (F-araNAD) was added with ATRA. F-araNAD is a cell impermeable known suicide substrate that inhibits CD38 ectoenzyme activity by forming a covalent adduct with E226 in the active pocket [23, 28]. CD38 has the potential to be internalized [29-32], including to the nucleus. Since the binding of F-araNAD to CD38 is covalent, then the internalized CD38 would be expected to retain the inhibitor. To verify the ability of F-araNAD to block CD38 enzymatic activity, we used the fluorometric NGD+ substrate assay. NGD+ is a NAD+ analog that can be cyclized by CD38 to cGDPR, a stable fluorescent product. We compared the fluorescence of untreated HL-60 cells with and without



**Figure 2.1 CD38 inhibitors do not affect ATRA-induced differentiation markers.** A: Cells were cultured for 48 hours with or without 1  $\mu$ M ATRA and cells were assayed for cGDPR production by NGD+ assay. The fold increase of cGDPR production was calculated by normalizing each sample to an arbitrary null control value of 1 (\* $p$  < 0.05). B: Membrane CD11b expression was measured by flow cytometry at indicated time points. (\* $p$  < 0.05 significantly higher than HL-60 cells that were not treated with ATRA.) C: HL-60 cells were treated or not with 1  $\mu$ M ATRA for 72 hours. Appropriate samples were incubated with 5  $\mu$ M F-araNAD. Cell cycle phase distribution was determined by flow cytometry with propidium iodide staining. (\* $p$  < 0.05 significantly higher than untreated G1/0 sample groups; # $p$  < 0.05 significantly lower than untreated S sample groups.) D: Cell density was measured using a hemocytometer and 0.2% Trypan blue exclusion staining.

NGD+ substrate and found no significant differences, indicating that untreated control cells had negligible ADPR cyclase activity (data not shown). We then compared the relative amount of cGDPR fluorescence generated by untreated cells, ATRA-treated HL-60 cells expressing CD38, or cells co-treated with ATRA plus 5  $\mu$ M F-araNAD (Fig. 2.1a). Fluorescence is reported in arbitrary units where 1 is the null background of control cells without CD38. While there was a significant difference in fluorescence between untreated cells and cells treated with ATRA, there were no apparent differences between control cells and co-treated cells. The inhibitor was therefore effective in crippling CD38 catalytic activity.

Next we determined the effects of CD38 inhibitors on ATRA-induced differentiation. ATRA treatment causes HL-60 cells to exhibit differentiation markers such as the  $\alpha$ -integrin receptor subunit CD11b. F-araNAD had no apparent effect on ATRA-induced CD11b expression, with or without ATRA treatment (Figure 2.1b).

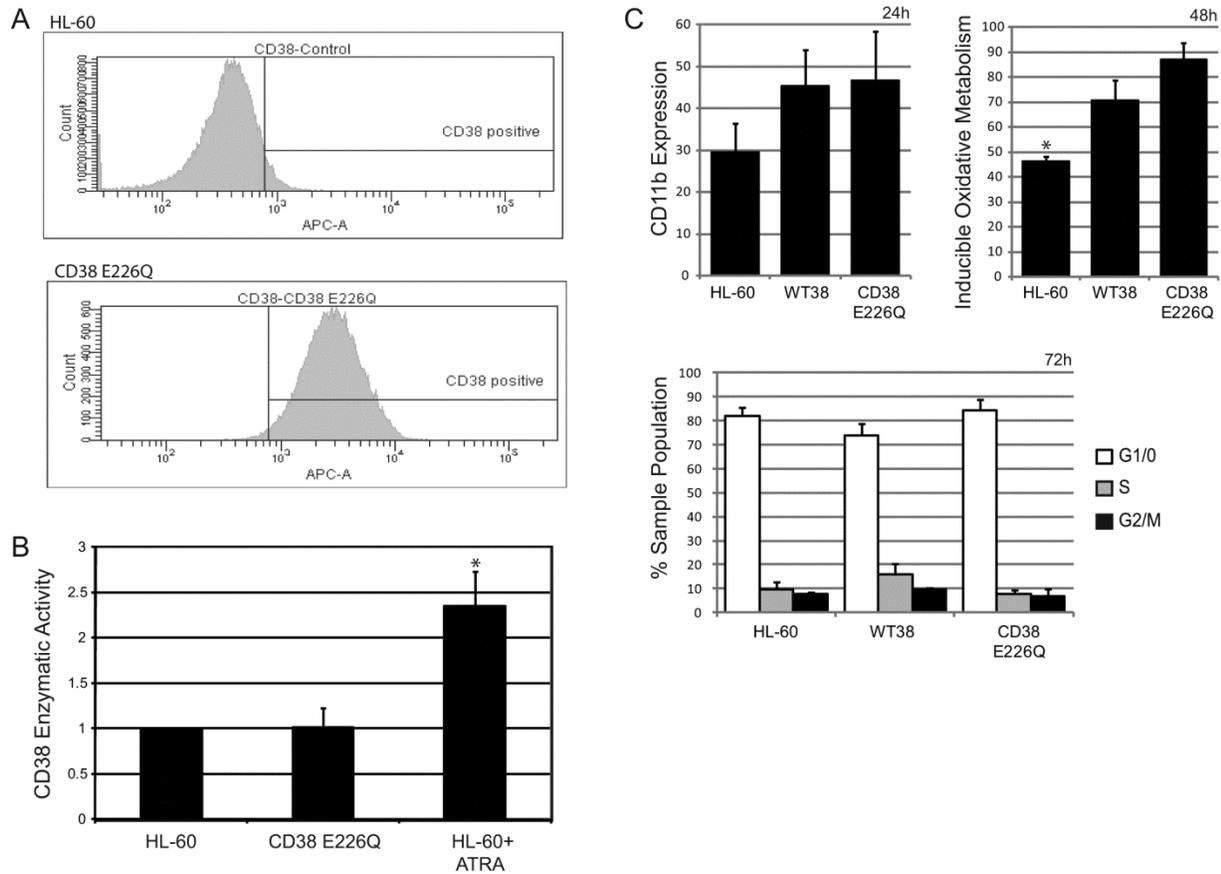
ATRA-treated HL-60 cells also undergo G1/0 enrichment, indicating cell cycle arrest. Cells were cultured with or without retinoic acid, and with or without 5  $\mu$ M F-araNAD for 72 hours. The proportion of G1/0 cells in neither ATRA-treated samples nor untreated samples was affected by inhibitor treatment (Figure 2.1c). Cell density was also measured during 72 hours to detect growth arrest (Figure 2.1d). ATRA-treated samples, regardless of F-araNAD treatment, show similar growth retardation at 72 hours. Untreated HL-60 cells and cells treated only with the inhibitor show continuous exponential growth. Therefore, inhibition of CD38 enzymatic activity by F-araNAD did not affect several markers of ATRA-induced myeloid differentiation. A second inhibitor, Arabinosyl-2'-fluoro-2'-deoxy nicotinamide mononucleotide (F-araNMN), was also tested and similarly failed to affect ATRA-induced differentiation and corroborated these results

(data not shown).

*2.4.2 An E226Q mutation does not alter CD38 effects on ATRA-induced differentiation or G1/0 arrest.* The experiments using pharmacological inhibition indicated that inhibiting the ectoenzyme activity of the endogenous CD38 did not affect induced differentiation, suggesting that the ectoenzyme activity is not critical. This gives rise to an anticipation that was tested. Since ectopic expression of wild-type CD38 promotes induced differentiation, then the enzymatically inactive [33] CD38 E226Q catalytic mutant would be expected to do the same if the ectoenzyme activity was not needed for this. Therefore, we ectopically expressed CD38 E226Q and investigated whether or not overexpression of CD38 E226Q was able to similarly drive ATRA induction.

We created cells stably transfected with the CD38 E226Q catalytic mutant and verified the mutation by sequencing. Expression of protein was confirmed by flow cytometry of immunostained cells (Figure 2.2a). Cell viability was unaffected by E226Q expression since cells showed normal growth, no increase in Trypan Blue exclusion staining, and no detectable sub-G1 population using cytometric propidium iodide staining (data not shown). Expression of CD38 after ATRA treatment was comparable in both WT38 and CD38 E226Q transfectants, allowing comparison without caveats due to achieving different expression levels (data not shown). The enzymatic activity for CD38 E226Q was tested using the NGD+ assay. cGDPR levels in E226Q cells were comparable to that of untreated HL-60 cells, showing that the point mutation was effective in crippling catalytic activity (Figure 2.2b). ATRA-treated HL-60 cells expressing CD38 were used as a positive control.

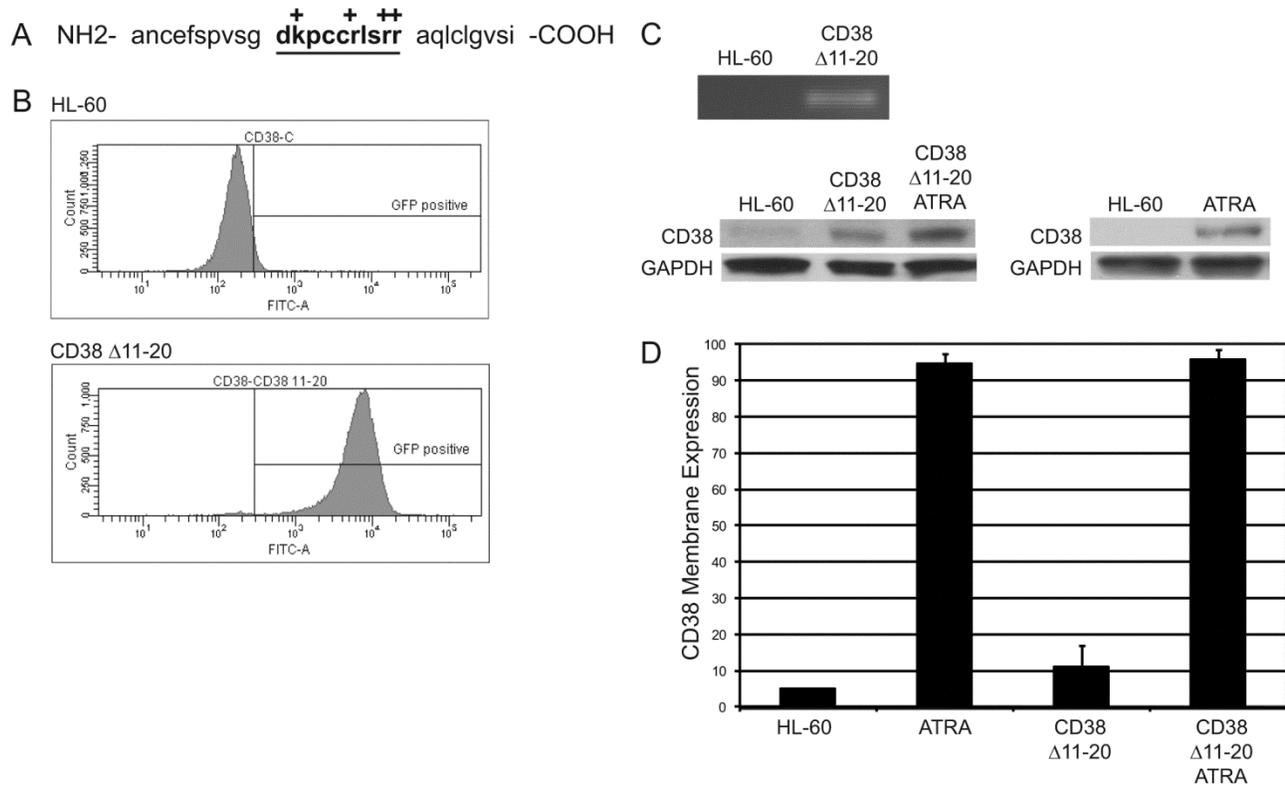
Cells stably transfected with wild-type CD38 (WT38) showed enhanced ATRA-induced



**Figure 2.2 CD38 E226Q is able to promote ATRA induction similar to wild-type CD38.** A: Flow cytometry immunostaining for CD38 in untreated HL-60 cells and CD38 E226Q stable transfectants. B: Cells were assayed for cGDPR production by NGD+ assay. The fold increase of cGDPR production was calculated by normalizing each sample to an arbitrary null control value of 1 (\* $p < 0.05$ ). C: Cells were treated for 24 hours with ATRA, and induced CD11b expression was measured by flow cytometry (top left); cells were treated for 48 hours with ATRA, and inducible oxidative metabolism was measured by DCF assay (top right; \* $p < 0.5$  significantly lower than WT38 and CD38 E226Q); cell lines were cultured with ATRA for 72 hours and cell cycle phase distribution was determined by nuclear propidium iodide staining (bottom). No significant differences ( $p < 0.05$ ) were detected between WT38 and CD38 E226Q samples in any of the above experiments.

differentiation [1]. To determine if CD38 E226Q and WT38 cells propelled differentiation similarly, we analyzed several markers to investigate whether or not the catalytic mutation had any affect. We first compared CD11b expression in response to ATRA in HL-60, WT38, and CD38 E226Q cells. After 24 hours, both WT38 and CD38 E226Q showed increased CD11b expression compared to ATRA-treated HL-60 cells (Figure 2.2c, top left). We then compared oxidative metabolism capability. After 48 hours ATRA-treated WT38 and CD38 E226Q showed a comparable increase in inducible oxidative metabolism, which was significantly higher than ATRA-treated HL-60 cells (Figure 2.2c, top right). These results suggested that both WT38 and CD38 E226Q expressing cells are similarly capable of enhancing functional differentiation. Finally, to determine if there were effects on cell cycle inhibition, we analyzed ATRA-induced G1/0 arrest. ATRA-treated HL-60, CD38 E226Q, and WT38 cells all showed similar G1/0 arrest by 72 hours (Figure 2.2c, bottom). CD38 E226Q transfected cells thus underwent differentiation indistinguishably from WT38 cells in response to ATRA. Like wild-type CD38, CD38 E226Q was also able to enhance CD11b expression and inducible oxidative metabolism compared to HL-60 cells. This confirms the anticipation of the earlier F-araNAD results.

*2.4.3 A cytosolic deletion prevents membrane expression in CD38 transfectants.* The CD38 cytosolic N-terminal tail is dispensable for signaling in pro-B cells [34, 35]. However, Moreno-Garcia et al. show that the cytosolic tail is important for CD38 homodimer stabilization and a truncation mutant decreases the half-life of membrane expression in murine B lymphocytes [36], which may be important for signaling. To assess the contribution of the short CD38 cytosolic tail to ATRA induction, we determined whether a deletion affected membrane expression of CD38 or ATRA-induced HL-60 differentiation. Ten amino acids were truncated in the cytosolic N terminus proximal to the transmembrane region (Figure 2.3a). These 10 residues were reported

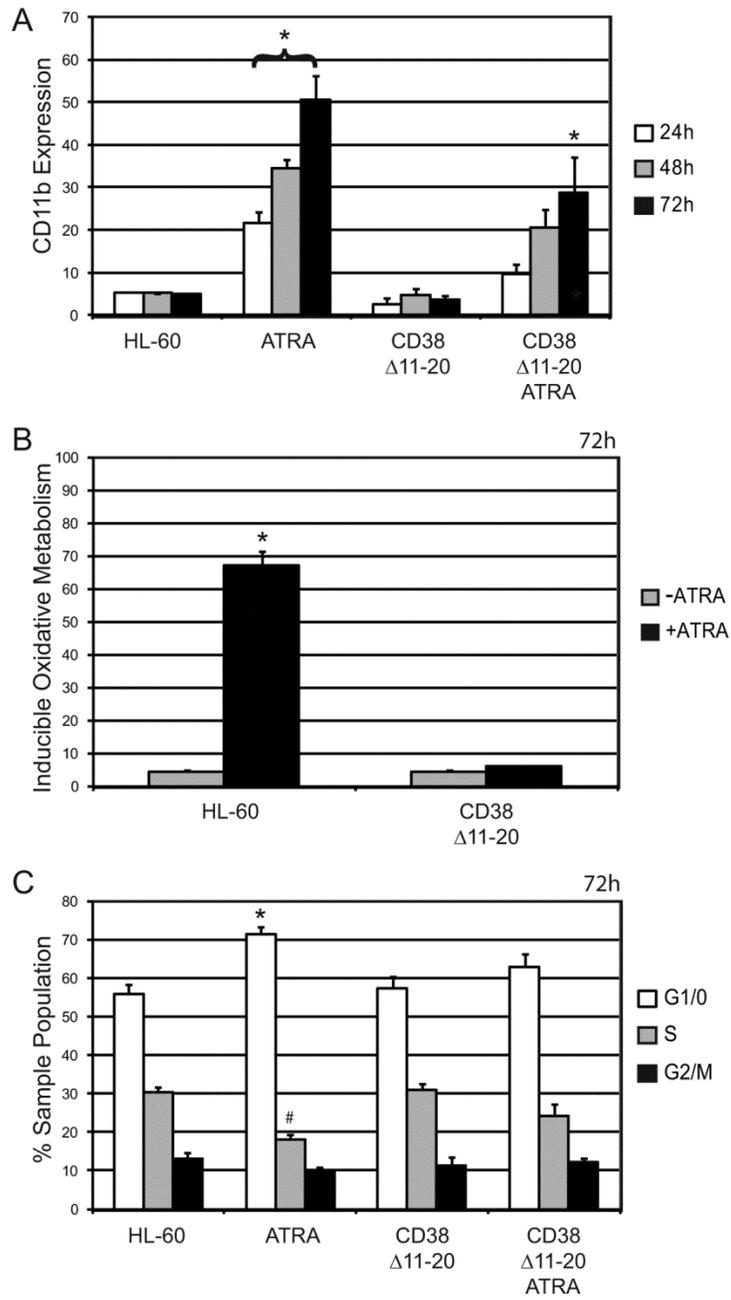


**Figure 2.3 A CD38 cytosolic deletion mutant does not express on the membrane.** A: CD38 N-terminal cytosolic and transmembrane amino acids are shown. Deleted amino acids are in bold and underlined. Positively charged amino acids are indicated with a +. B: GFP-positive population in HL-60 and CD38  $\Delta$ 11-20 cells. C: mRNA from HL-60 cells and CD38  $\Delta$ 11-20 was subjected to reverse-transcriptase PCR for CD38 expression and visualized on an agarose gel (top). Western blot for total CD38 protein in CD38  $\Delta$ 11-20 and HL-60 cells, with and without 48 hours of ATRA treatment (bottom). Untreated HL-60 cells were used as a negative control for showing CD38  $\Delta$ 11-20 expression in transfectants. D: Membrane expression of CD38 was measured after 24 hours of culture by immunostaining and flow cytometry.

to be necessary for interaction with Lck, a Src family kinase, in T cell signaling events [9, 37] and were considered good candidates for interactions with other proteins in different cell lines.

Stable transfectants expressing the CD38  $\Delta$ 11-20 deletion were isolated by FACS sorting using a GFP marker co-expressed from a CD38 $\Delta$ 11-20/GFP bicistronic transcript with an IRES element (Figure 2.3b). The deletion was confirmed by mRNA isolation followed by reverse transcriptase PCR and gel analysis, and protein expression was confirmed by Western blot (Figure 2.3c). Cell viability was unaffected by CD38  $\Delta$ 11-20 overexpression since cells showed normal growth, no increase in Trypan Blue exclusion staining, and no detectable sub-G1 population using cytometric propidium iodide staining (data not shown). Interestingly, although ATRA-treated HL-60 cells expressing wild-type CD38 were able to be membrane-labeled with antibody, cells expressing CD38  $\Delta$ 11-20 showed minimal labeling and no significant difference in membrane expression compared to the untreated control (Figure 2.3d). The lack of CD38  $\Delta$ 11-20 membrane expression could reflect the loss of the positively charged residues in the transmembrane-proximal cytosolic region, which may be essential for proper membrane insertion and orientation. This is consistent with studies that report CD38 mutant proteins with truncated cytosolic regions show significantly reduced membrane expression and half-lives compared to wild-type protein [36]. After CD38  $\Delta$ 11-20 transfectants were treated with ATRA, membrane expression of CD38 was comparable to that of ATRA-treated HL-60 cells consistent with endogenous CD38 expression.

*2.4.4 CD38  $\Delta$ 11-20 cripples ATRA-induced differentiation induction.* To determine the effect of the  $\Delta$ 11-20 deletion on ATRA induction, we conducted flow cytometric analyses of HL-60 cells and CD38  $\Delta$ 11-20 transfectants to assess their ability to undergo differentiation. Compared to



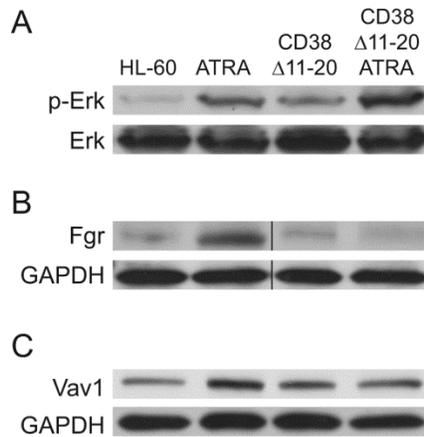
**Figure 2.4 CD38  $\Delta$ 11-20 expression interrupts differentiation markers.** A: Membrane CD11b expression was measured by immunostaining and flow cytometry at indicated time points. (\*p<0.05 significantly higher than untreated HL-60 cells at the same time point.) B: After 72 hours, inducible oxidative metabolism was measured by flow cytometry using the DCF assay. (\*p<0.05 significantly higher than untreated HL-60 cells.) C: Cells were cultured for 72 hours with or without ATRA and cell cycle phase distribution was determined by nuclear propidium iodide staining. (\*p<0.05 G1/0 phase was significantly enriched compared to untreated HL-60 cells; #=p<0.05 S phase was significantly lower than untreated HL-60 cells.)

ATRA-treated HL-60 cells, treated CD38  $\Delta$ 11-20 transfectants showed significantly less CD11b expression at 24 and 48 hours, and decreased expression at 72 hours (Figure 2.4a). This indicated that expression of the  $\Delta$ 11-20 deletion decreased ATRA-induced expression of CD11b.

Next we analyzed inducible oxidative metabolism after ATRA treatment. For HL-60 cells, the percentage capable of producing reactive oxygen species (ROS) increased to approximately 70% at 72 hours compared to the untreated control (Figure 2.4b). However, CD38  $\Delta$ 11-20 transfectants showed a lack of oxidative metabolism capability, indicating CD38  $\Delta$ 11-20 transfectants are unable to undergo the inducible respiratory burst characteristic of mature myeloid cells.

After 72 hours of ATRA treatment, HL-60 cells showed significant G1/0 DNA enrichment compared to the untreated counterparts (Figure 2.4c). However, after ATRA treatment the percentage of CD38  $\Delta$ 11-20 cells in G1/0 was not significantly different than untreated HL-60 cells, indicative of crippled ATRA-induced G1/0 arrest. Together, these results indicate that CD38  $\Delta$ 11-20 is able to inhibit terminal differentiation as evidenced by crippled G1/0 enrichment and oxidative metabolism, and decreased expression of CD11b.

*2.4.5 CD38  $\Delta$ 11-20 expression affects signaling proteins regulated by ATRA.* MAPK signaling can result in cell proliferation or arrest and differentiation, depending on receptor type and signaling longevity [18, 38]. During ATRA-induced HL-60 differentiation, a protracted Raf/MEK/ERK signal is required, and inhibitors of Raf and MEK block HL-60 leukemic cell maturation [19, 20]. Overexpression of wild-type CD38 drives MAPK signaling on its own and results in sustained, upregulated ERK signaling without ATRA treatment [1]. This motivated interest in whether the CD38  $\Delta$ 11-20 mutant is capable of driving MAPK signaling.



**Figure 2.5 CD38  $\Delta$ 11-20 expression modulates some ATRA-regulated signaling molecules.**

A: HL-60 cells or CD38  $\Delta$ 11-20 cells were treated or not with ATRA for 48 hours and Western blot analysis for ERK phosphorylation was performed with total ERK used as a loading control.

B: Western blot for Fgr expression after 48 hours of ATRA treatment or untreated culture. GAPDH was used as a loading control.

C: Western blot of Vav1 expression after 48 hours of ATRA treatment or untreated culture. GAPDH was used as a loading control.

HL-60 cells and CD38  $\Delta$ 11-20 transfectants were ATRA-treated or left untreated for 48 hours, and phosphorylated ERK1/2 was assessed (Figure 2.5a). The CD38  $\Delta$ 11-20 transfectants showed enhanced ERK activation compared to HL-60 cells. ATRA increased activated ERK in both cases with CD38  $\Delta$ 11-20 cells still exceeding HL-60. Hence the CD38  $\Delta$ 11-20 cells were still capable of producing a persistent cellular MAPK signal, but it did not propel ATRA-induced differentiation.

While CD38  $\Delta$ 11-20 appears to be capable of generating a MAPK signal, its inhibitory effect on ATRA-induced differentiation suggests that this signaling differs from that of wild-type CD38. One such possibility is disruption of MAPK regulators such as Src family kinases and the guanine nucleotide exchange factor Vav1 [39-42]. ATRA upregulates Fgr and Vav1 [43, 44], and Vav1 interacts with CD38 through a c-Cbl containing complex [14]. This motivated interest in the effect of CD38  $\Delta$ 11-20 on Fgr and Vav1.

Fgr upregulation is proposed to prevent apoptosis and promote granulocytic differentiation [43, 45]. Therefore, Fgr may be important for ATRA-induced signaling mechanisms and cell survival through maturation. Treated HL-60 cells showed upregulated expression of Fgr while CD38  $\Delta$ 11-20 cells did not (Figure 2.5b). Premature cell death was not observed with Trypan Blue exclusion staining nor was the presence of sub-G1 populations detectable by flow cytometry (data not shown), indicating the cells were not experiencing elevated apoptosis. These results implied that the expression of CD38  $\Delta$ 11-20 interfered with the expected ATRA-induced Fgr upregulation.

Vav1, a guanine nucleotide exchange factor (GEF) for the Rho family of Ras-related GTPases, becomes upregulated [44] after ATRA treatment in HL-60 cells and can be phosphorylated by

Fgr [46]. After 48 hours of ATRA, HL-60 cells showed a modest increase in total Vav1 expression (Figure 2.5c). However, there was no change in Vav1 expression in CD38  $\Delta$ 11-20 cells. These results indicated that ATRA-induced Vav1 upregulation was also defective when CD38  $\Delta$ 11-20 was expressed.

## 2.5 Discussion

CD38 is a membrane-expressed protein that is used as a prognostic indicator in leukemia. While it is often considered a negative marker in chronic lymphocytic leukemia (CLL), the consequences of CD38 expression are somewhat enigmatic in acute myelogenous leukemia (AML). Because CD38 is associated with signaling mechanisms that may induce proliferation, differentiation, or apoptosis [22, 27, 47] the role of CD38 in cell survival and terminal arrest remains ambiguous. This could reflect the diversity of its functions and capabilities.

CD38 is known to have both enzymatic and receptor functions. The extracellular domain is able to catalyze the formation of cADPR and NAADP<sup>+</sup>, which are both calcium-mobilizing second messengers. CD38 also participates in a variety of signaling events by serving as a ligand-activated receptor and by forming lateral associations with other proteins at the cell membrane. Here we attempt to segregate several of these functions and determine which are important for ATRA-induced myeloid differentiation in HL-60 human leukemia cells. We find that a cytosolic deletion mutant (CD38  $\Delta$ 11-20) which does not membrane express interrupted differentiation, as evidenced by a lack oxidative metabolism, insignificant growth arrest, and decreased CD11b expression. Expression of CD38  $\Delta$ 11-20 also caused failure to upregulate markers including Fgr and Vav1 which are induced by ATRA and may be MAPK modulators. In contrast, crippling enzymatic activity by inhibitors had no effect on ATRA-induced myeloid differentiation. Also,

cells expressing catalytically inactive CD38 showed similar enhanced differentiation as cells expressing wild-type CD38.

CD38  $\Delta$ 11-20 transfectants failed to show ATRA-induced G1/0 enrichment and oxidative metabolism, suggesting that they are unable to undergo respiratory burst and growth arrest characteristic of terminal differentiation. Src family tyrosine kinases such as Fgr, which is expressed in differentiated myeloid cells after ATRA treatment, mediate respiratory burst [46, 48]. Fgr failed to up-regulate in ATRA-treated CD38  $\Delta$ 11-20 cells. The transfectants also showed decreased ATRA-induced expression of CD11b, an  $\alpha$ -integrin receptor subunit. Treating HL-60 cells with molecules that upregulate CD11b increases inducible oxidative metabolism [6, 49]. Membrane-localized Fgr plays a role in integrin receptor signaling [50], and integrin signaling may be important for hematopoietic myelo-monocytic differentiation [51, 52]. This suggests that the compromised induced expression of both Fgr and CD11b may decrease the oxidative metabolism capability in CD38  $\Delta$ 11-20 cells that is normally characteristic of differentiated myelocytes.

CD38  $\Delta$ 11-20 expression also disrupted ATRA-induced expression of Vav1, which is tyrosine phosphorylated by Fgr [46]. Vav1 is a Rho/Rac GEF that also regulates PI3K activity during myeloid differentiation [45, 53] and MAPK signaling in lymphocytes [39, 40]. Overexpression of Vav1 enhances myeloid differentiation and coincides with nuclear translocation of its tyrosine-phosphorylated form and nucleoskeleton rearrangement [44, 54]. ATRA-treated HL-60 cells showed an increase in Vav1 expression compared to untreated cells, while CD38  $\Delta$ 11-20 transfectants did not.

The MAPK signaling cascade is also activated in HL-60 cells after ATRA treatment and is

required for differentiation progression [19, 55]. In CD38  $\Delta$ 11-20 transfectants ERK phosphorylation was enhanced after 48 hours of ATRA treatment but cell differentiation was inhibited. These results indicate that while MAPK signaling is necessary, it is not sufficient in itself for ATRA-induced differentiation. It is possible that CD38  $\Delta$ 11-20 is changing the character of the MAPK cascade and affecting cellular outcomes by disrupting MAPK signaling regulators, which may include Vav1, Fgr, and other molecules. The MAPK network employs numerous adaptor, scaffolding, and inhibitory accessory proteins that regulate signaling; and direct MAPK signaling effectors such as Raf, MEK, and ERK may also serve as scaffolds, adaptors, and inhibitors themselves to finely tune the outcome of MAPK signaling (reviewed in [56]). Therefore CD38  $\Delta$ 11-20, or ATRA-modulated signaling molecules that are not upregulated in CD38  $\Delta$ 11-20 cells, could be aberrantly interacting with or affecting the regulation of signaling networks such as MAPK that are important for myeloid differentiation induction. It is also possible that CD38  $\Delta$ 11-20 itself may disrupt protein-protein interactions with ATRA-induced, endogenous membrane CD38 and its downstream effectors, thus crippling wild-type signaling. Further research will be needed to elucidate how the CD38  $\Delta$ 11-20 deletion mutant inhibited differentiation, but the discovery that it appears to act as a dominant negative is noteworthy. While the results in this study support the notion that CD38 plays a direct role in differentiation, we cannot rule out the possibility that the proteins affected by CD38  $\Delta$ 11-20 expression, such as CD11b, Fgr, and Vav1, either act independently or as accessory signaling proteins, and their disruption interferes with differentiation.

In contrast, crippling enzymatic cADPR catalytic activity, either by treatment with a specific small molecule inhibitor or by site-directed mutagenesis, does not impair ATRA-induced differentiation progression. WT38 and CD38 E226Q transfectants that overexpress wild-type or

catalytically inactive protein, respectively, show similar enhanced differentiation compared to parental HL-60 cells. Regulation of ATRA-induced differentiation by CD38 thus does not appear to depend on its ectoenzyme activity. The results of other studies are consistent with this premise. CD38 enzymatic activity and receptor functions are uncoupled in a murine pro-B leukemic cell line. Receptor signaling cascades show dependence on downstream tyrosine kinase activity but are independent of ADP-ribosyl cyclase and NAD-glycohydrolase mechanisms [27, 57].

In conclusion, these results suggest that membrane-expressed CD38 may be required for the regulation and activation of downstream signaling mechanisms induced by ATRA treatment, which a non-membrane, cytosolic deletion mutant blocks. CD38 apparently promotes ATRA-induced myeloid differentiation through its receptor but not ecto-enzymatic functions.

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## CHAPTER III

### THE CD38 $\Delta$ 11-20 MUTANT CRIPPLES ATRA-INDUCED GRANULOCYTIC MATURATION BUT NOT 1,25-DIHYDROXYVITAMIN D3-INDUCED MONOCYTIC DIFFERENTIATION

#### 3.1 Abstract

HL-60 human myeloid leukemia cells are bipotent and differentiate to neutrophils or monocytes after treatment with all-*trans* retinoic acid (ATRA) or 1,25-dihydroxyvitamin D<sub>3</sub>, respectively. A previous report shows that cells expressing a CD38 cytosolic mutation (CD38  $\Delta$ 11-20) fail to differentiate after ATRA treatment, evidenced by crippled upregulation of differentiation markers such as CD11b and failure to G<sub>1</sub>/0 arrest. Here we report that CD38  $\Delta$ 11-20 cells are capable of monocytic differentiation induced by D<sub>3</sub>, evidenced by expression of CD11b and G<sub>1</sub>/0 arrest that is similar to wild-type parental HL-60 cells. These results show that CD38  $\Delta$ 11-20 cells are responsive to D<sub>3</sub> but not to ATRA, and suggests that membrane-expressed, wild-type CD38 is important for ATRA induction but not monocytic maturation propelled by D<sub>3</sub>.

#### 3.2 Introduction

Bipotent HL-60 myeloid leukemia cells differentiate along the granulocytic lineage when treated with all-*trans* retinoic acid (ATRA) and undergo monocytic maturation when treated with 1,25-dihydroxyvitamin D<sub>3</sub> (D<sub>3</sub>). Treatment with either agent causes increases in CD11b, CD14, and CD38 expression, and G<sub>1</sub>/0 arrest. D<sub>3</sub> treatment results in more CD11b and CD14 expression and less CD38 expression compared to ATRA [1, 2]. D<sub>3</sub> and ATRA induction both depend on MAPK signaling through the c-Raf/MEK/ERK axis [3-6]. CD38 and one of its partner

molecules, c-Cbl, both drive MAPK signaling and differentiation when overexpressed [7-9]. Therefore CD38 and its accessory molecules may propel signaling necessary for both ATRA and D3 induction.

A stable transfectant cell line expressing a CD38 cytosolic tail truncation that is not membrane expressed (CD38  $\Delta$ 11-20) shows defective ATRA-induced differentiation. This is evidenced by decreased G1/0 arrest and oxidative metabolism capability, and crippled upregulation of differentiation markers including CD11b, Vav1, and Fgr [10]. The data suggest that wild-type membrane CD38 is required for ATRA-induced myeloid differentiation and motivated interest in whether or not CD38  $\Delta$ 11-20 cells were also defective in D3-induced monocytic differentiation. We confirmed previous data showing that CD38  $\Delta$ 11-20 cells treated with ATRA exhibited crippled CD11b expression and G1/0 arrest. However, after D3 treatment CD38  $\Delta$ 11-20 transfectants showed CD11b expression and G1/0 arrest similar to treated parental HL-60 cells. This indicates that wild-type membrane CD38 is required for ATRA-induced granulocytic differentiation but not for D3-induced monocytic maturation, and suggests that the efficacy of D3 treatment does not depend on CD38 expression or its receptor signaling functions.

### **3.3 Materials and Methods**

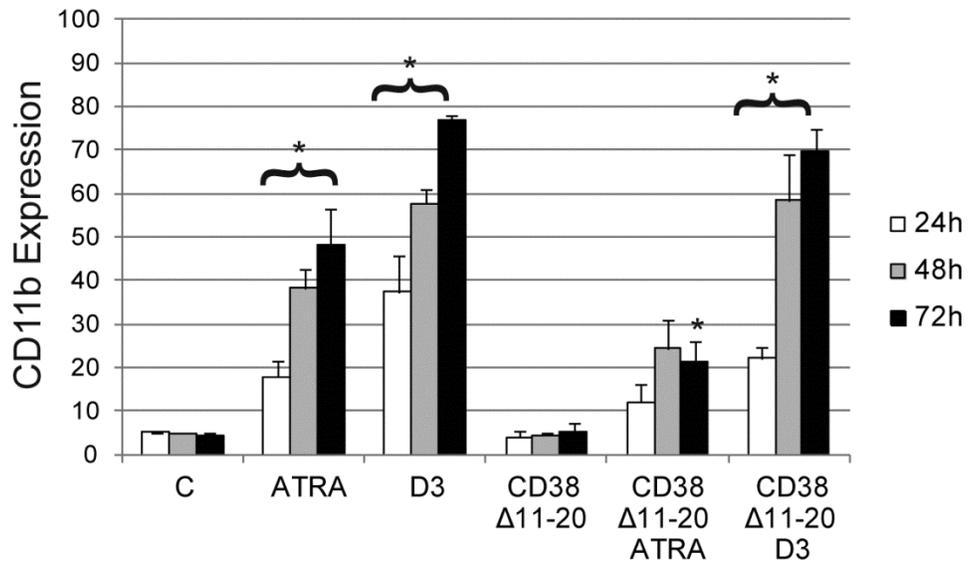
*Cell culture.* HL-60 human myeloblastic leukemia cells and CD38  $\Delta$ 11-20 stable transfectant cells were grown in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum purchased from Invitrogen (Carlsbad, CA) in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. CD38  $\Delta$ 11-20 stable transfectants were created as described [10]. ATRA was purchased from Sigma (St. Louis, MO) and solubilized in ethanol. Cells were treated with a final concentration of 1  $\mu$ M. 1,25-dihydroxyvitamin D3 was purchased from Sigma and solubilized in ethanol. Cells were

treated with a final concentration of 5  $\mu$ M.

*Flow Cytometry.* Immunostaining for CD11b with antibody from BD Pharmingen (San Jose, CA) was performed as previously described using a Becton Dickinson LSR II flow cytometer (San Jose, CA) [8]. Gating was set to exclude 95% of the negative control. Propidium iodide (PI) cell cycle analysis was performed as previously described [8].

### **3.4 Results**

*3.4.1 CD38  $\Delta$ 11-20 cells show CD11b expression comparable to wild-type HL-60 cells after treatment with D3 but not ATRA.* First we evaluated expression of the differentiation marker and  $\alpha$ -integrin receptor CD11b. CD11b is detectable within 24 hours of ATRA- or D3-treatment and increases in expression over time, with D3 showing enhanced expression compared to ATRA. Since CD38  $\Delta$ 11-20 transfectants display crippled CD11b expression after ATRA, we evaluated if this was also the case after D3 treatment. We evaluated CD11b by flow cytometry after the indicated treatments and time points (Figure 3.1). Untreated HL-60 and CD38  $\Delta$ 11-20 cells show negligible CD11b at all time points. ATRA and D3 treated HL-60 cells show elevated expression within 24 hours of treatment; an approximate 20% and 40% increase compared to the untreated control, respectively. Expression continues to increase over time, with D3 showing enhanced expression as anticipated for wild-type HL-60 cells. However, while ATRA-treated CD38  $\Delta$ 11-20 cells show crippled CD11b expression, D3 treatment results in CD11b levels comparable to D3-treated parental HL-60 cells after 48 hours. Therefore unlike ATRA, D3 treatment is able to facilitate upregulation of the differentiation marker CD11b in CD38  $\Delta$ 11-20 cells.



**Figure 3.1 CD38  $\Delta$ 11-20 cells treated with D3 show CD11b expression similar to wild-type HL-60 cells.** Cells were immunostained for CD11b at the indicated time points. Gating was set to exclude 95% of the negative control (untreated HL-60 cells) for each time point. (\* $p < 0.05$  significantly higher than untreated HL-60 cells at the same time point.)

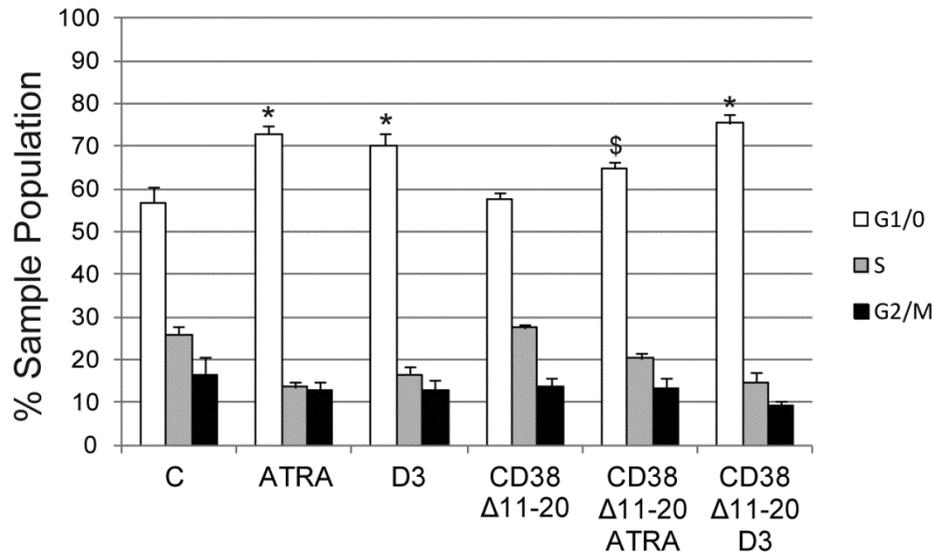
*3.4.2 CD38  $\Delta$ 11-20 cells treated with D3 are capable of cell cycle arrest.* HL-60 and CD38  $\Delta$ 11-20 cells were either left untreated or treated with D3 or ATRA, and after 72 hours G1/0 enrichment was evaluated (Figure 3.2). HL-60 cells treated with ATRA or D3, and CD38  $\Delta$ 11-20 cells treated with D3, show significant G1/0 enrichment compared to untreated controls (Figure 3.2). However CD38  $\Delta$ 11-20 cells treated with ATRA have a significantly lower percentage of cells in G1/0 than all other ATRA- or D3-treated samples. Therefore, CD38  $\Delta$ 11-20 cells were capable of cell cycle arrest after D3 treatment but not ATRA.

### **3.5 Discussion**

CD38 is a leukocyte antigen known to propel ATRA-induced myeloid maturation and MAPK signaling that is necessary for differentiation [7]. Wild-type, membrane-expressed CD38 appears to be required for ATRA's efficacy, since CD38-targeted siRNA interferes with differentiation and a cytosolic mutant (CD38  $\Delta$ 11-20) that fails to membrane express likewise cripples the effects of ATRA [10, 11]. However whether or not CD38 is important for D3-induced monocytic differentiation is unknown. We used CD38  $\Delta$ 11-20 stable transfectants that show little response to ATRA and treated them with D3 to evaluate if they reacted to a monocytic inducer.

Surprisingly, we found that CD38  $\Delta$ 11-20 cells responded to D3 similar to wild-type HL-60 cells, and were capable of CD11b upregulation and G1/0 arrest. Therefore, expression of a mutant non-membrane CD38 protein that disrupts ATRA induction did not interfere with the effects of D3. These results indicate that CD38 and its receptor signaling functions may not be important for D3-induced differentiation.

The results also suggest that early cell signaling events during the first 24 hours of ATRA



**Figure 3.2 CD38  $\Delta$ 11-20 cells treated with D3 show G1/0 cell cycle arrest comparable to wild-type HL-60 cells.** Cells were treated as indicated for 72 hours and cell cycle was measured using flow cytometry and propidium iodide staining. (\* $p < 0.05$  G1/0 phase was significantly enriched compared to untreated HL-60 cells; \$= $p < 0.05$  G1/0 phase significantly lower than all other samples that received ATRA or D3 treatment.)

treatment, such as those propelled by CD38, are lineage-specific. A previous report proposes that early cellular events and signaling are not specific to lineage, since 24 hours of ATRA treatment followed by 24 hours of D3 results in monocytic differentiation and vice versa [12]. In contrast, the data presented here indicates that early events, including the expression of CD38 and the signaling cascades it regulates, may be important or not depending on lineage.

The mechanism by which CD38  $\Delta$ 11-20 cripples ATRA induction requires additional investigation, and could illuminate protein interactions and signaling pathways that distinguish granulocytic maturation from monocytic. For example, different molecules could be involved in lineage-specific MAPK signaling propagation. CD38 drives MAPK signaling, and both drive granulocytic differentiation. While D3 also requires a persistent MAPK signal, that signal may be regulated by a different ensemble of molecules. Also, proteins associated with membrane-expressed CD38 and drive ATRA-induced differentiation (which could be misregulated or sequestered by CD38  $\Delta$ 11-20) may not be important for monocytic differentiation induced by D3. In this case CD38  $\Delta$ 11-20 may not impede the effects of D3. CD38-modulated proteins could include c-Cbl, which associates with CD38 and also drives MAPK signaling, and c-Cbl partners such as Vav1 and SLP-76 that also appear to regulate granulocytic differentiation. Future research may focus on the identification of CD38-associated molecules that are important for ATRA induction, and elucidate if they impinge on D3-induced differentiation.

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## CHAPTER IV

### LOSS OF DIFFERENTIATION PRECOMMITMENT MEMORY CORRESPONDS TO THE LOSS OF CD38 AND p-ERK PARENT-TO-DAUGHTER HERITABILITY

#### 4.1 Abstract

Continuous treatment of HL-60 acute myeloblastic leukemia cells with all-*trans* retinoic acid (ATRA) causes terminal differentiation along the granulocytic lineage. During the first 24 hours cells show early markers of phenotypic conversion including ERK phosphorylation and expression of CD38, which is known to drive MAPK signaling. Previous results show that after 48 hours cells are committed to differentiation and show signs of late myeloid maturation including G1/0 arrest and inducible oxidative metabolism. A preceding study reports that 24-hour exposure to ATRA followed by drug removal results in the acquisition of a semi-stable precommitment memory, where abbreviated re-exposure to ATRA induces late differentiation markers. This memory precommitment is lost after three cell division cycles. Here we report the loss of precommitment memory temporally corresponds with the loss of CD38 and p-ERK parent-to-daughter heritability. After three division cycles following ATRA removal cells showed inherited CD38 and p-ERK but lost the ability to confer expression to daughter cells, since after four divisions cells showed crippled CD38 expression and p-ERK comparable to control. This suggests that CD38 may drive MAPK signaling which contributes to the maintenance of a heritable precommitment state where cells are “primed” for differentiation.

#### 4.2 Introduction

Continuous treatment of HL-60 acute myelogenous leukemia cells with ATRA results in

terminal differentiation along the granulocytic lineage. After two cell division cycles (48 hours) of ATRA exposure, HL-60 cells are committed to terminal differentiation evidenced by G1/0 arrest and later signs of phenotypic conversion including inducible oxidative metabolism [1, 2]. During the first 24 hours of ATRA exposure cells exhibit early markers of induced differentiation such as CD38 and ERK phosphorylation, but no evidence of G1/0 arrest or other late markers. HL-60 cells that have undergone 24 hours of ATRA exposure are characterized as precommitted and are “primed” to differentiate. For example, if ATRA is withdrawn after one cell division (24 hours), HL-60 cells will continue to grow exponentially and fail to terminally differentiate. However reintroduction of ATRA during up to three cell division cycles (24, 48, or 72 hours) post-ATRA withdrawal results in significant differentiation after one cell cycle, characterized by G1/0 arrest and inducible respiratory burst [1-5]. Therefore cells seem to retain a “commitment memory” after the initial ATRA exposure which is heritable but labile, since after three divisions (96 hours) cells are no longer primed and require the full 48 hours of ATRA retreatment to show evidence of late differentiation.

The semi-stable precommitment state is characterized by structural changes to the nucleus [1, 2], and one report suggests it is not lineage-specific [6]. This study shows that sequential treatment of HL-60 cells with the monocytic inducer 1,25-dihydroxyvitamin D3 (D3) for 24 hours followed by ATRA (a granulocytic inducer) results in granulocytic differentiation and vice versa, suggesting that the differentiation lineage is decided by late exposure to the inducing agent. However data presented in Chapter 3 indicate that some early events, including CD38-driven signaling, may have lineage-specific roles.

Since early treatment over the course of one cell division primes cells for terminal differentiation, it is important to elucidate which signaling molecules may regulate the

maintenance of precommitment memory. CD38 is an early marker of ATRA-induced differentiation (detectable 6 hours after treatment) that drives MAPK signaling when overexpressed [7]. MAPK signaling evidenced by modifications of the Raf/MEK/ERK axis is reported to be required for ATRA effectiveness, since inhibitors of MEK and c-Raf suppress differentiation [8, 9]. CD38 itself is also thought to play a causal role in ATRA induction since overexpression propels myeloid maturation, while a cytosolic mutant that is not membrane-expressed or siRNA targeting CD38 cripples the effects of ATRA treatment [10, 11]. There is also evidence that CD38 may be involved in a positive feedback loop that sustains MAPK signaling (Tasseff et al., unpublished).

We tracked changes in the expression of CD38 and ERK phosphorylation status over time after “priming” HL-60 cells with ATRA for 24 hours and then withdrawing treatment. We found that CD38 expression exceeded 80% of the untreated control for three cell divisions after ATRA removal, indicating that the proliferating cells sustained CD38 expression. When cells had completed four division cycles (96 hours post-ATRA removal) CD38 was still expressed at levels that were approximately 75% of the untreated control but these cells were not capable of giving rise to daughter cells with high CD38 expression. This corresponds to the loss of precommitment memory, since cells reintroduced to ATRA at 96 hours require a full 48 hours of re-treatment to show late differentiation markers. After the fifth division CD38 expression dropped to levels comparable to control. Likewise, heritable ERK phosphorylation was persistent for 96 hours after ATRA removal, but then fell to levels similar to the untreated control, indicating that parent-to-daughter p-ERK heritability was lost. Therefore, the significant decrease in the ability of parental HL-60 cells to sustain CD38 and p-ERK expression followed a time course similar to HL-60 precommitment loss. This suggests CD38 drives MAPK signaling to

maintain a cellular ATRA exposure memory and the ability to remain “primed” for differentiation.

### **4.3 Materials and Methods**

*Cell culture.* HL-60 cells were grown in RPMI 1640 with 1% antibiotic/antimycotic from Invitrogen (Carlsbad, CA) and treated with 1  $\mu$ M ATRA as previously described [10]. For precommitment experiments cells were treated with ATRA for 24 hours, washed 3x to remove ATRA, and reseeded every 24 hours at  $0.2 \times 10^6$ /mL in ATRA-free media as described.

*Antibodies and reagents.* Antibody for cytometric analysis of CD38 was purchased from BD Pharmingen (San Jose, CA). p-Erk1/2 and GAPDH were purchased from Cell Signaling (Danvers, MA). Protease and phosphatase inhibitors, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). M-PER Mammalian Protein Extraction Reagent lysis buffer was purchased from Pierce (Rockford, IL).

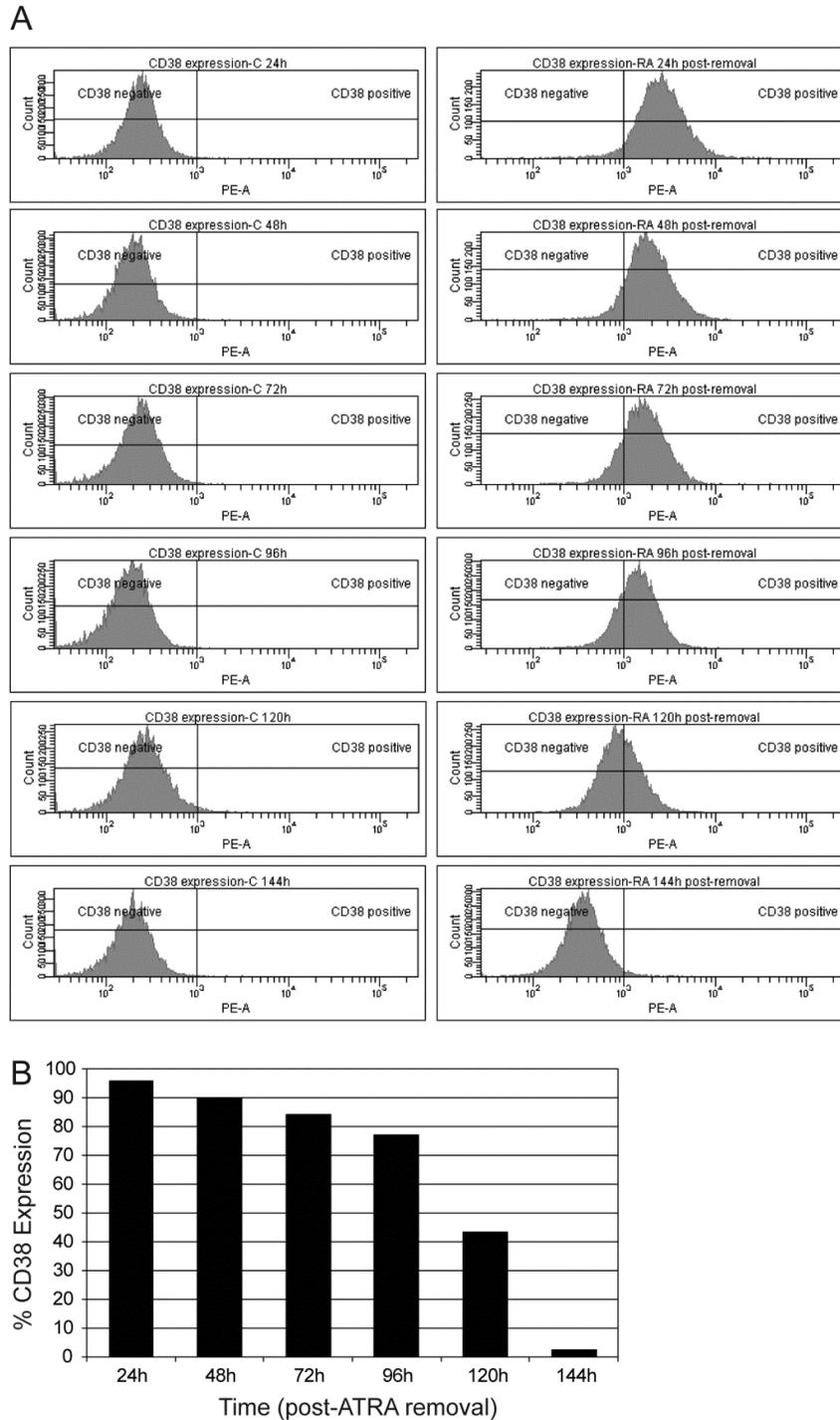
*Flow cytometric phenotypic analysis.* Immunostaining for CD38 was performed by treating  $1 \times 10^6$  or  $0.5 \times 10^6$  cells with antibody (concentration per manufacturer’s instructions) for one hour in PBS at 37°C. Fluorescence was detected using a Becton Dickinson LSR II flow cytometer (San Jose, CA) as previously described [12]. Gating for positive cells was set to exclude 95% of the negative control.

*Western blot analysis.* Cells were lysed and subjected to standard SDS-PAGE analysis as previously described [10].

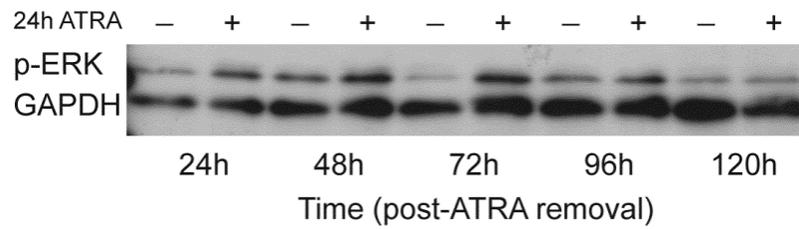
## 4.4 Results

*4.4.1 CD38 heritability is synchronized with ATRA precommitment sensitivity.* Since cells treated with ATRA for 24 hours are primed for differentiation and show a precommitment sensitivity, we monitored changes in CD38 expression after ATRA withdrawal to evaluate if there were time-dependent similarities to precommitment memory maintenance. We treated HL-60 cells with ATRA for 24 hours and then reseeded them in ATRA-free media. HL-60 expression of CD38 remained high through the first four cell divisions (96 hours) after ATRA removal: approximately 95% of the negative control at 24 hours, 90% at 48 hours, 80% at 72 hours, and 75% at 96 hours (Figure 4.1a&b). Interestingly after three divisions (96 hours) parent cells are no longer able to impart high CD38 expression to daughter cells, since at 120 hours post-ATRA removal CD38 expression significantly decreased to 40% indicating dilution by cell division. CD38 expression was similar to control after five divisions. The loss of CD38 parent-to-daughter heritability therefore corresponded temporally with the loss of precommitment memory.

*4.4.2 Parent-to-daughter conferral of ERK phosphorylation is concurrent with loss of CD38 heritability and precommitment memory.* Since MAPK signaling is required for ATRA-induced differentiation and CD38 drives ERK activation, we monitored ERK phosphorylation status after a 24 hour pulse exposure to ATRA followed by removal. Similar to CD38 expression, cells showed inherited ERK phosphorylation through the first four divisions, but after that parental cells were unable to confer p-ERK expression to daughter cells (Figure 4.2). Therefore, after four divisions (120 hours) post-ATRA removal cells no longer showed high CD38 or p-ERK inheritance. This raises the possibility that the significant decrease in CD38 expression may have contributed to the loss of ERK phosphorylation.



**Figure 4.1 Parent cells do not confer high CD38 expression to daughter cells 96 hours post-ATRA removal.** A: Flow cytometry histograms for CD38 show decreasing expression after ATRA removal. B: CD38-positive expression post-ATRA removal. Gating was set to exclude 95% of the negative control.



**Figure 4.2 Loss of heritable ERK phosphorylation coincides with loss of precommitment memory.** Western blot for p-ERK at indicated time points. GAPDH was used as a loading control.

## Discussion

CD38 expression and ERK phosphorylation are early markers of ATRA-induced myeloid differentiation, and CD38 drives MAPK signaling [7]. Within 24 hours of ATRA exposure HL-60 cells also develop a precommitment memory and are “primed” for differentiation. This precommitted state persists for three division cycles post-ATRA withdrawal, evidenced by detectable late differentiation markers after only 24 hours of ATRA reintroduction. Therefore cells that are re-exposed to ATRA 24, 48, or 72 hours after removal are “primed” for differentiation. However, if cells are re-exposed 96 hours after the initial ATRA treatment, differentiation requires a full 48 hours of ATRA reintroduction, similar to untreated cells [1]. This phenomenon suggests there are early events after ATRA treatment that are important for maintenance of a labile precommitment memory. This motivated interest in whether changes in the expression of early ATRA-induced markers, such as CD38 and p-ERK (which may be driven by CD38), show parallelism to the gain and loss of a precommitted state.

We found that the loss of the parental cells’ ability to sustain high heritable expression of CD38 or phosphorylated ERK corresponded with the loss of precommitment memory. For three division cycles after ATRA withdrawal, parent cells were able to impart CD38 and p-ERK expression to daughter cells. Although high CD38 expression was inherited through four division cycles after ATRA exposure, after the third division parent cells could not give rise to daughter cells with CD38 high expression. This coincided with the loss of p-ERK parent-to-daughter heritability.

These results suggest that CD38 signaling, which drives ERK phosphorylation, may support the maintenance of the precommitment memory state. CD38 signaling is reported to be important for

ATRA effectiveness, since siRNA targeted against CD38 and a cytosolic mutant that is not membrane expressed (CD38 $\Delta$ 11-20) both interrupt ATRA-induced differentiation [10, 11]. Similarly, MAPK signaling inhibitors cripple ATRA induction [8, 9]. The data are consistent with a model whereby ATRA induces CD38 expression to generate a MAPK signal. This sustains a precommitment memory during three cell divisions, which regulates the maintenance of a state where HL-60 cells are “primed” for differentiation.

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## CHAPTER V

### LYN KINASE ACTIVITY REGULATES ATRA-INDUCED AND CD38-PROPELLED PHOSPHORYLATION OF c-CBL AND p85 PI3K

#### 5.1 Abstract

The leukocyte antigen CD38 is expressed after all-*trans*-retinoic acid (ATRA) 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 all three of these proteins complex with Lyn, a Src family kinase (SFK) upregulated by ATRA. We used the SFK inhibitor PP2 to evaluate if CD38-driven signaling depends on Lyn kinase activity. Cells treated with ATRA for 48 hours followed by one hour of PP2 incubation show SFK/Lyn kinase inhibition. We observed that Lyn inhibition blocks c-Cbl and p85/p55 PI3K phosphorylation driven by the CD38 agonist IB4 in ATRA-treated HL-60 cells and untreated CD38+ transfectants. In contrast, cells treated with ATRA and PP2 at the same time and then cultured for 48 hours did not show Lyn inhibition, therefore ATRA protected Lyn from the effects of PP2. 48 hours of co-treatment also preserved CD38-stimulated c-Cbl and p85/p55 PI3K phosphorylation indicating Lyn kinase activity is necessary for these events. Another SFK inhibitor (dasatinib) which blocks Lyn activity with ATRA co-treatment prevented ATRA-induced c-Cbl phosphorylation and crippled p85 PI3K phosphorylation, indicating Lyn kinase activity is important for ATRA-propelled events which may be regulated by CD38. We also found that the 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 ATRA-induced differentiation.

## 5.2 Introduction

All-*trans* retinoic acid (ATRA) 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 ATRA-propelled myeloid differentiation in non-APL cells. Molecules and signaling pathways that confer ATRA responsiveness in HL-60 cells may be important in elucidating how a non-APL leukemia cell can be induced to differentiate by ATRA, and may ultimately provide knowledge that could expand the use of ATRA as a therapeutic agent.

CD38 is a leukocyte antigen that is an early marker of ATRA induction and drives differentiation when overexpressed [1]. CD38 has enzymatic activity that generates the Ca<sup>2+</sup> mobilizing compounds NAADP<sup>+</sup> and cADPR. It also has receptor functions that drive cell signaling including the phosphorylation of c-Cbl, ERK, and the p85 PI3K regulatory subunit [1-8].

Enzymatic activity and receptor/signaling functions can operate independently [9-11]. For example, CD38 metabolic activity is unnecessary for ATRA-induced differentiation while the receptor function associated with membrane-expressed CD38 is required [12]. In addition, siRNA targeting CD38 cripples differentiation [13]. These reports suggest that CD38-driven signaling is important for ATRA-driven myeloid maturation. Therefore, it is of interest to identify CD38-associated signaling molecules and how they may regulate ATRA efficacy. Such knowledge may indicate targets for therapeutic intervention.

CD38 forms a complex with c-Cbl [14, 15] and CD38 agonist ligand interaction results in c-Cbl phosphorylation [2]. c-Cbl is an E3 ubiquitin ligase and adaptor molecule that, like CD38, promotes MAPK signaling and ATRA-induced differentiation when overexpressed [2, 14, 15]. This suggests that the c-Cbl/CD38 interaction may cooperatively drive MAPK signaling and other aspects of ATRA therapy. This is consistent with a report that a c-Cbl tyrosine kinase binding (TKB) domain mutant (G306E) that does not bind CD38 also fails to drive MAPK signaling and differentiation [15].

c-Cbl is known to interact with the guanine nucleotide exchange factor (GEF) Vav1, the SLP-76 adaptor, and, like CD38, the p85 regulatory subunit of PI3K [14-17]. c-Cbl, SLP-76, and Vav1 protein expression and p85 PI3K activity are upregulated during granulocytic maturation [18-22]. These four proteins also form complexes in myeloid cells after ATRA treatment. For example, Vav1 associates with PI3K and may facilitate the characteristic nucleoskeleton remodeling that occurs with ATRA treatment in HL-60 and NB4 cells [23, 24]. Consistent with this, downmodulation of Vav1 impedes induced myeloid maturation and nucleoskeleton remodeling, and affects differentiation-related protein expression [22]. 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 ATRA treatment Vav1/c-Cbl complexes are detectable in the cytosol, while Vav1/SLP-76 interactions are predominant in nuclei [23]. SLP-76 is also upregulated after ATRA and forms a complex with c-Cbl [15]. Co-expression of SLP-76 with CSF-1/c-FMS enhances ATRA-induced ERK activation, G0 cell cycle arrest, and a number of additional differentiation markers [20].

CD38 agonist ligation by the m(Abs) IB4 and T16 induces phosphorylation of the p85 regulatory subunit of PI3K and is associated with normal and leukemic B cell growth suppression [25-27]. Consistent with this, PI3K inhibitors relieved CD38-mediated growth suppression in ATRA-treated HL-60 cells [28], which suggests a PI3K-modulated CD38 feedback loop.

CD38 also drives transient MAPK activation after agonist ligation, which is orchestrated by the Raf/MEK/ERK axis [5, 6]. Transient or protracted signaling from this cascade can lead to either cell proliferation or differentiation respectively [29], and sustained MAPK signaling characterizes ATRA-induced differentiation [30, 31]. CD38 overexpression results in persistent ERK phosphorylation, therefore CD38 appears capable of propagating a transient or sustained MAPK signal.

Lyn and other SFKs are known to be modulated by ATRA 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 [32]. 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 [33]. Likewise, in lymphoblastoid B-cell membrane rafts CD38 is associated with Lyn and modulates cell signaling [34].

SFK inhibitors are known to enhance aspects of ATRA induction, including expression of CD11b and other myeloid maturation markers [35, 36]. A recent study reported that dasatinib, which inhibits Lyn kinase activity alone and with ATRA co-administration, enhances differentiation (Congleton et al., in press). However the inhibitor PP2, which inhibits Lyn alone but does not block kinase activity with ATRA co-treatment, shows a more significant

enhancement of ATRA-induced differentiation than dasatinib. Therefore, Lyn kinase activity may function to drive some aspects of differentiation.

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 ATRA provides therapeutic benefit. Clarification of pathways that confer ATRA 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, ATRA has shown some promise in treating reproductive leiomyomas by modulating the PI3K signaling cascade [37].

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 no effect on ERK phosphorylation, but was able to completely abrogate c-Cbl and p85 PI3K phosphorylation driven by the CD38 agonist IB4 [27, 38]. We used CD38+ stable transfectants and ATRA-treated HL-60 cells to evaluate if the effects of the inhibitor were associated with either ATRA 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 ATRA and PP2 followed by 48 hours of culture protects Lyn kinase activity from PP2 inhibition and significantly enhances differentiation (Congleton et al., in press). Protecting Lyn kinase activity using ATRA/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 ATRA co-treatment, prevented ATRA-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 ATRA 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/ATRA co-treatment that result in differentiation enhancement.

### **5.3 Materials and Methods**

*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  $\mu$ M ATRA as previously described [12]. PP2 from EMD Chemicals (Gibbstown, NJ) was solubilized in dimethyl sulfoxide (DMSO) at 10 mM. Cells were treated with a final concentration of 10  $\mu$ M 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 hemacytometer and trypan blue exclusion.

*Antibodies and reagents.* Protein A/G beads used for immunoprecipitation, 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, 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).

*Construction of CD38+ stable transfectants.* CD38 knock-in plasmid construction and transfection were performed as previously described [1]. 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.

*Western blot analysis and immunoprecipitation.* For immunoprecipitation experiments, cells were lysed as previously described [12]. 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 [12].

*FRET (fluorescence resonance energy transfer).* Cells were harvested, fixed, and permeabilized as previously described [15]. Cells were resuspended in 200  $\mu$ l of PBS containing 5  $\mu$ l 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 [15]. 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.

*Signaling experiments.* For signaling experiments with Lyn kinase inhibition cells were cultured for 48 hours with 1  $\mu$ M ATRA, and then washed twice with serum-free RPMI 1640 media. Appropriate samples were incubated with 10  $\mu$ M PP2 for one hour and all samples were incubated at 37°C with constant rotation. The indicated samples were then treated with 5  $\mu$ M IB4 for the time points as shown. For samples with ATRA/PP2 co-administration cells were cultured for 48 hours with 1  $\mu$ M ATRA and 10  $\mu$ M PP2. Signaling experiments were performed as described above for IB4 treatment, but the one hour PP2 pre-incubation was omitted.

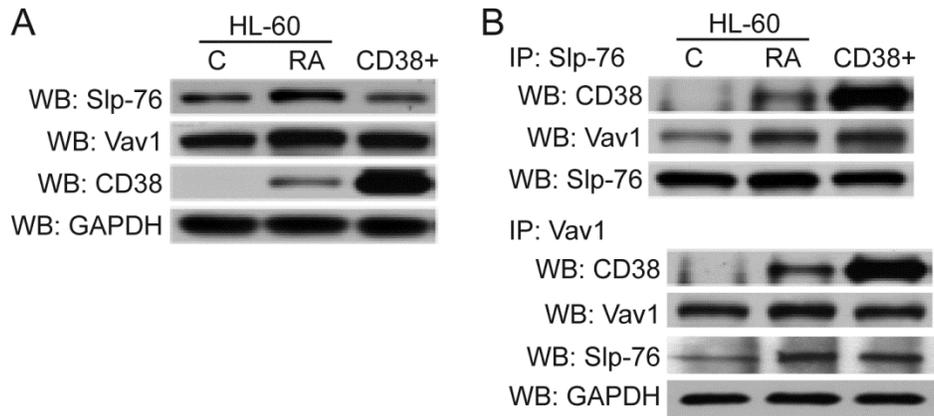
*Statistics.* Three independent repeats were conducted in all experiments. Error bars represent the standard error. The student's t-test function in MS Excel was used to analyze the data.

## 5.4 Results

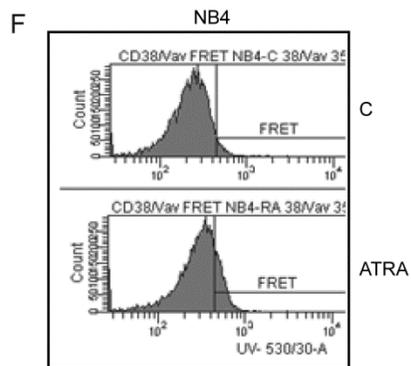
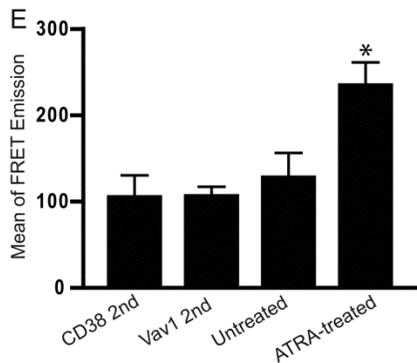
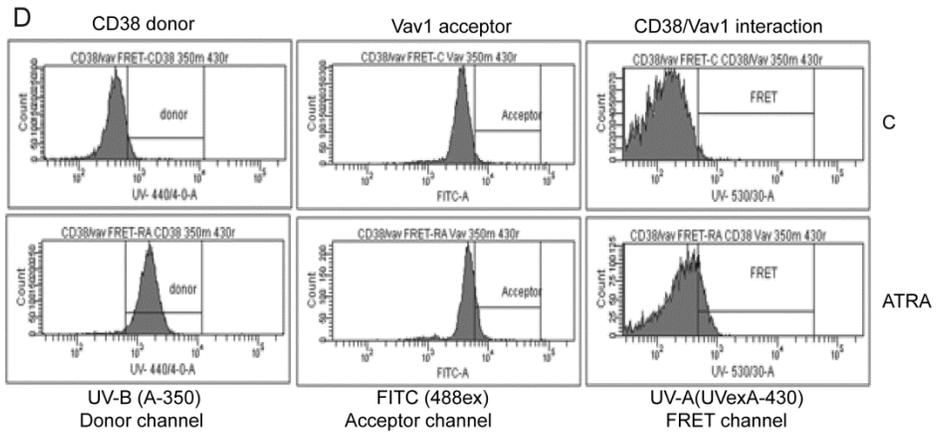
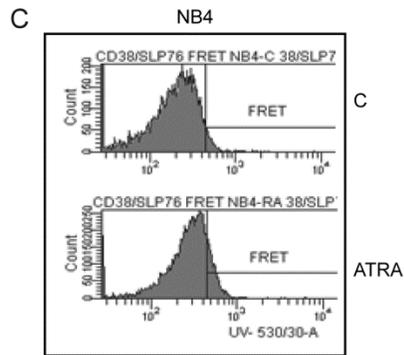
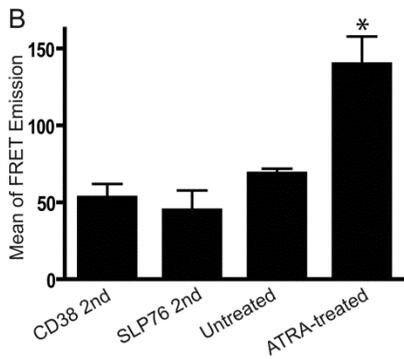
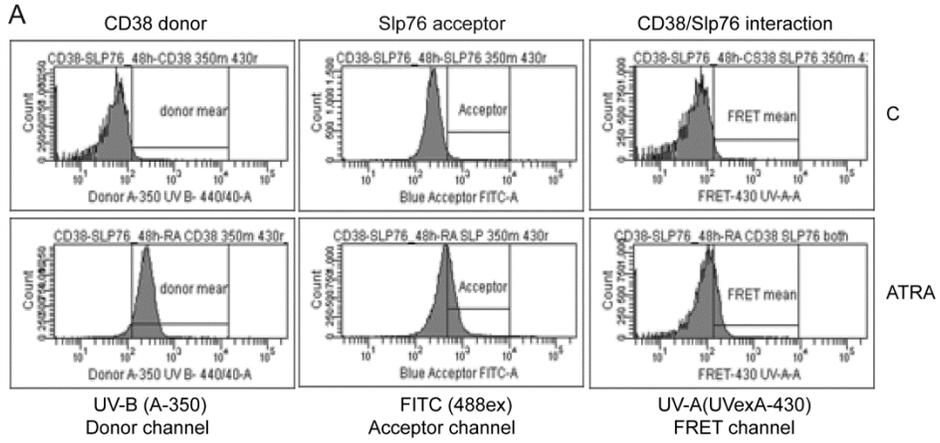
*5.4.1 CD38 interacts with the ATRA-regulated proteins Vav1 and SLP-76.* We first determined if there was interaction between CD38 and SLP-76 or Vav1, and investigated whether or not ectopically expressing CD38 in the absence of ATRA affected these interactions. Therefore we could compare effects that were dependent on ATRA treatment versus CD38 expression alone. Western blotting for total protein showed that SLP-76, Vav1, and CD38 were all upregulated by ATRA, with CD38 expression showing dependence on ATRA treatment (Figure 5.1a). CD38 overexpression in stable transfectants (CD38+) did not significantly increase Vav1 or SLP-76, indicating that upregulated expression of these proteins was dependent on ATRA.

Immunoprecipitation experiments show that ATRA-induced CD38 was able to complex with SLP-76 and Vav1, and CD38+ transfectants showed increased interaction (Figure 5.1b). ATRA 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.

*5.4.2 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 acute promyelocytic leukemia (APL) t(15,17) translocation to show that these interactions are not specific to HL-60 cells [39, 40]. As expected, CD38, SLP-76, and Vav1 were ATRA-upregulated as indicated in donor and acceptor channels



**Figure 5.1 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 ATRA 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).



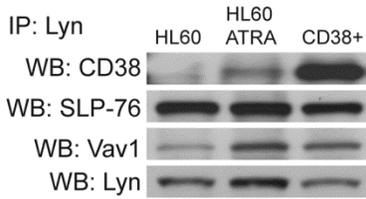
**Figure 5.2 FRET corroborates the CD38/Vav1/SLP-76 interaction.** A: Flow cytometry FRET histograms confirm 48 hours of ATRA upregulates CD38 and SLP-76 expression and their interaction in HL-60 cells. B: Graph showing means of CD38/SLP-76 FRET emission in HL-60 cells. C: CD38/SLP-76 FRET interaction 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.

(Figures 5.2a&d). FRET signals between CD38 and SLP-76 were observed in HL-60 and NB4 cells after 48 hours of ATRA treatment (Figures 5.2a,b,c) but not in untreated cells that did not express CD38. Likewise we detected interaction between CD38 and Vav1 in ATRA-treated HL-60 and NB4 cells (Figures 5.2d,e,f).

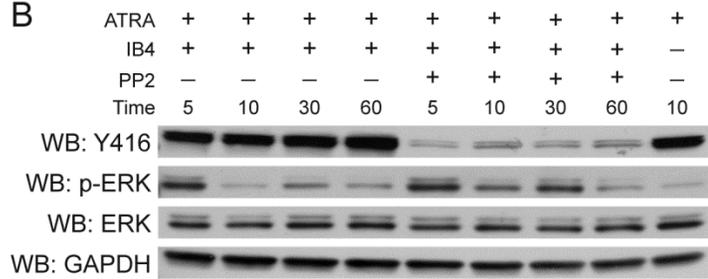
Together, the results from co-immunoprecipitation and FRET experiments demonstrated that ATRA-treated HL-60 and NB4 cells showed interaction among CD38, SLP-76, and Vav1, and indicated that CD38 facilitated a SLP-76/Vav1/CD38 complex. These results prompted interest in CD38/Vav1/SLP-76 interactions with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

*5.4.3 Lyn interacts with CD38, SLP-76, and Vav1, and kinase inhibition affects CD38-stimulated signaling.* Lyn is upregulated by ATRA and may modulate induction therapy since siRNA against Lyn interferes with differentiation [41]. Lyn also interacts with CD38 to promote signaling [3, 33, 34]. Therefore, we investigated if Lyn participated 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 5.3a). ATRA-induced CD38 complexed with Lyn, and untreated CD38+ cells showed significantly increased interaction, indicating that CD38 alone facilitates binding. ATRA 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.

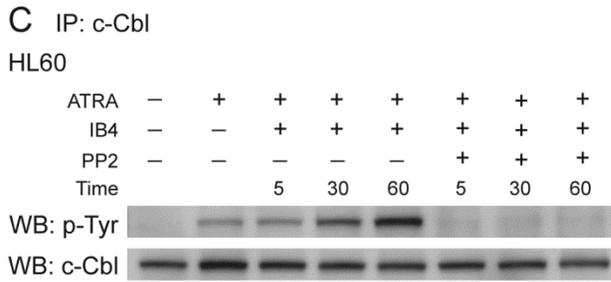
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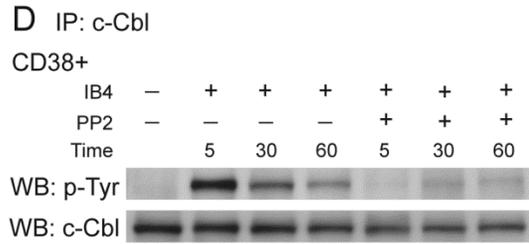
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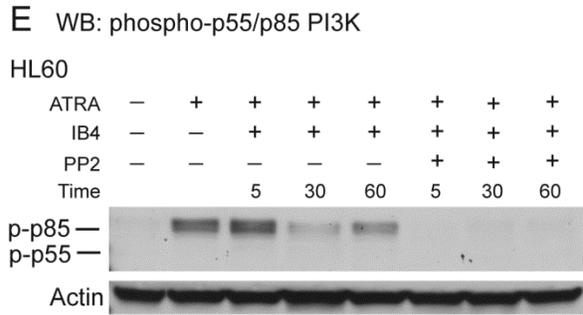
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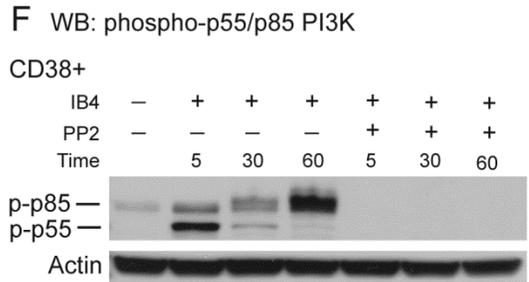
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**E**



**F**



**Figure 5.3 Lyn binds CD38, SLP-76, and Vav1 and its kinase activity regulates CD38**

**signaling.** A: Immunoprecipitation 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<sup>+</sup> cells. The IP:Lyn/WB:Lyn blot shows protein loading. B: Western blots for SFK/Lyn kinase activity in ATRA-treated HL-60 cells using a pan-Y416 that detects active site phosphorylation in all SKF 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. C: 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 D: c-Cbl tyrosine phosphorylation in CD38<sup>+</sup> transfectants in the absence of ATRA treated as indicated with PP2 for one hour and then stimulated with IB4. E: Western blot for phospho-p55/p85 PI3K in HL-60 cells treated as indicated. F: Western blot for phospho-p55/p85 PI3K in CD38<sup>+</sup> transfectants treated as indicated. Actin shows protein loading in pY-p55/p85 PI3K blots.

Since CD38, SLP-76, and Vav1 also interact with c-Cbl [14, 15], we probed for interaction between c-Cbl and Lyn but were unable to detect any significant evidence of association. We also immunoprecipitated Fgr, another SFK upregulated by ATRA, 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 [35, 42]. 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 the CD38 m(Ab) agonist IB4. Here, IB4 was used as a tool to stimulate CD38 signaling.

We first confirmed that PP2 was effective in crippling SFK activity in HL-60 cells treated with ATRA 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 the CD38 agonist ligand 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 5.3b). We found that PP2 was able to inhibit Lyn/SFK activity after IB4 stimulation in ATRA-treated cells and that IB4 ligation did not increase Lyn/SFK kinase activity, consistent with a previous report [27].

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 [32, 34]. We therefore evaluated whether the inhibitor affected transient IB4-induced ERK phosphorylation (Figure 5.3b). Although PP2 was able to inhibit SFK/Lyn kinase activity, it had no significant effect on transient ERK phosphorylation after IB4 stimulation of CD38. 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 5.3c). c-Cbl became phosphorylated after ATRA, and IB4 further stimulated phosphorylation. However, pre-incubation with PP2 for one hour was able to abrogate c-Cbl phosphorylation during all time points, indicating that Lyn activity is necessary for ATRA- 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 ATRA or CD38 expression alone (Fig. 5.3d). We found that PP2 was still able to block c-Cbl phosphorylation induced by IB4, showing the inhibitor blocked CD38-driven signaling independent of ATRA. It is noteworthy that the kinetics of c-Cbl phosphorylation induced by IB4 were different in CD38+ transfectants compared to ATRA-treated HL-60 cells, suggesting ATRA may temporally regulate modification of c-Cbl.

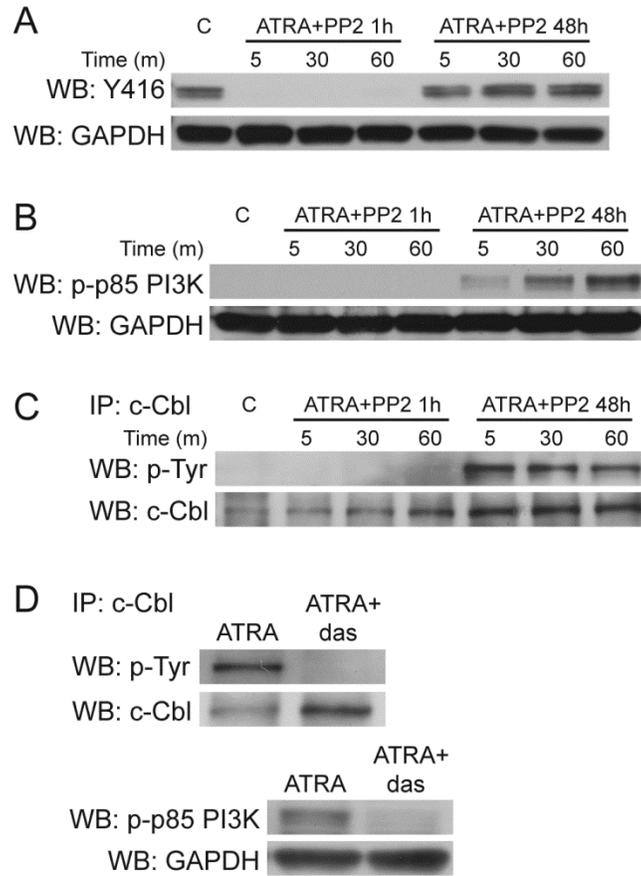
We also evaluated the effects of PP2 on p85 PI3K regulatory subunit phosphorylation. In HL-60 cells ATRA induced phosphorylation of the p85 PI3K subunit after 48 hours (Figure 5.3e). IB4 did not result in a significant increase in phosphorylation but this may be because ATRA is already stimulating p-p85 PI3K, and additional phosphorylation could not be achieved. Like pY-c-Cbl, PP2 was able to block ATRA-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 ATRA (Figure 5.3f). 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 ATRA-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 ATRA-treated HL-60 cells and CD38+ transfectants. PP2 did not affect CD38-propelled ERK phosphorylation, indicating that the signaling cascade that results in p-ERK is independent of Lyn kinase activity.

*5.4.4 Early PP2 treatment co-administered with ATRA protects Lyn kinase activity and permits CD38-driven c-Cbl and p85 PI3K phosphorylation.* After 48 hours of ATRA, one hour of PP2 incubation inhibits Lyn activity (Figure 5.3). However, if PP2 treatment occurs concurrently with ATRA and cells are then cultured for 48 hours, Lyn kinase activity is protected (Congleton et al., in press). The mechanism by which ATRA protects Lyn kinase activity from inhibition by PP2 is not known. Since one-hour PP2 incubation with ATRA-treated cells blocks SFK/Lyn kinase activity but PP2/ATRA 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 ATRA were able to protect Lyn activity. We compared Y416 phosphorylation in cells that were co-treated with ATRA and PP2 simultaneously and then cultured for 48 hours to cells that received only ATRA for 48 hours followed by PP2 incubation for one hour (Figure 5.4a). All ATRA-treated samples were treated with IB4 for the indicated time points to stimulate CD38. As expected, cells treated with ATRA and then later incubated with PP2 showed no Y416 phosphorylation, while cells co-treated with ATRA and PP2 for 48 hours permitted SFK/Lyn kinase activity.

We then evaluated if protecting Lyn activity correlated with the ability of ATRA and IB4 to stimulate CD38-driven c-Cbl and p85 PI3K phosphorylation. Confirming the results in Figure 5.3, one hour of PP2 incubation in ATRA-treated cells blocked p85 PI3K and c-Cbl



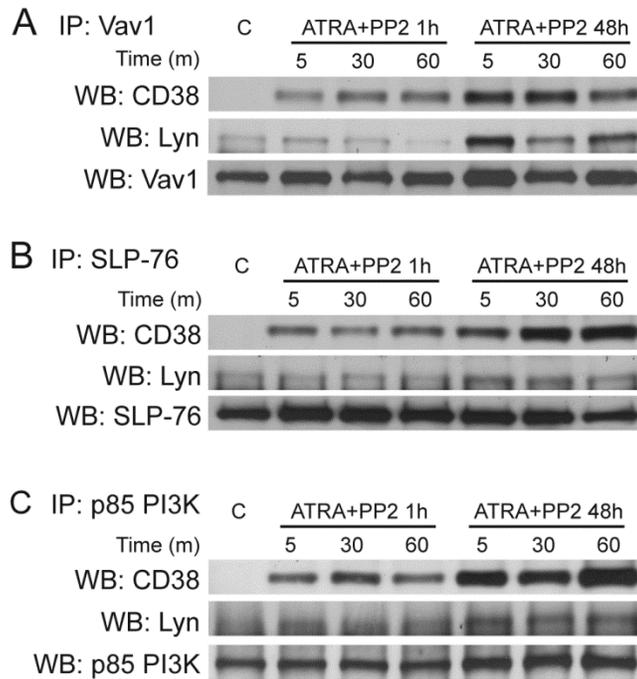
**Figure 5.4 Protecting Lyn kinase activity preserves CD38-stimulated c-Cbl and p85 PI3K phosphorylation.** A: Western blotting shows Y416 inhibition in HL-60 cells treated with ATRA for 48 hours followed by one hour of PP2 incubation, but activation after 48 hours of ATRA/PP2 co-treatment in culture. 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).

phosphorylation in IB4-stimulated cells (Figures 5.4b&c). However, 48 hour co-treatment with ATRA and PP2 permitted ATRA- and IB4-induced c-Cbl and p85 PI3K phosphorylation.

Finally we used dasatinib, which unlike PP2 abrogates Lyn activity with ATRA co-administration (Congleton et al., in press), to evaluate if kinase inhibition blocked ATRA-induced c-Cbl and p85 PI3K phosphorylation (Figure 5.4d). We found that dasatinib blocked c-Cbl phosphorylation and significantly decreased p85 PI3K phosphorylation, suggesting that ATRA-induced pY-c-Cbl requires Lyn activity and ATRA-upregulated p85 PI3K activity is largely dependent on Lyn.

Therefore, SFK/Lyn kinase activity regulated ATRA-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 ATRA-induced molecules, specifically SLP-76 and Vav1, were affected by intact or lost Lyn kinase activity.

*5.4.5 PP2 inhibition of c-Cbl and p85 PI3K phosphorylation coincides with decreased SLP-76/CD38/Lyn, Vav1/CD38/Lyn, and p85 PI3K/CD38/Lyn 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 ATRA treatment or received simultaneous PP2/ATRA treatment for 48 hours. All ATRA-treated samples were stimulated by IB4 to elicit CD38 signaling. We anticipated that an ATRA-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 Vav1/CD38 and Vav1/Lyn interaction (Figure 5.5a). This suggests that these three molecules constitute part



**Figure 5.5 Lyn inhibition disrupts interactions among CD38-associated proteins. A:** Immunoprecipitation of Vav1 shows changes in CD38 and Lyn interaction after treatments as indicated in HL-60 cells. The Vav1 blot shows protein loading. **B:** Immunoprecipitation of SLP-76 shows changes in CD38 and Lyn interaction after treatments as indicated. The SLP-76 blot shows protein loading. **C:** Immunoprecipitation of p85 PI3K shows changes in CD38 and Lyn interaction after treatments as indicated. The p85 PI3K blot shows protein loading. HL-60 cells in A, B and C that received ATRA treatment were cultured for 48 hours.

of a signaling complex that regulates an ATRA-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 5.5b). 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 Vav1/CD38/Lyn and SLP-76/CD38/Lyn. This suggests that a putative signaling complex including CD38, Vav1, SLP-76, and active Lyn regulates CD38-driven signaling which characterizes ATRA-induced differentiation. Finally we observed that Lyn inhibition decreased p85 PI3K/CD38/Lyn interaction (Figure 5.5c), suggesting that CD38 facilitates a Lyn kinase-containing complex that is directly responsible for p85 PI3K phosphorylation.

## **5.5 Discussion**

ATRA therapy is successful in treating t(15,17) positive acute promyelocytic leukemia (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 ATRA 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 ATRA responsiveness could broaden its usefulness in other diseases and aid in identifying new therapeutic molecular targets.

CD38 is a leukocyte enzyme and receptor that drives MAPK signaling and differentiation when overexpressed [1]. It has important functions in ATRA induction, since siRNA targeting CD38

interferes with differentiation, and a CD38 mutant (CD38  $\Delta$ 11-20) that is not membrane-expressed also cripples ATRA effectiveness [12, 13]. Identifying signaling pathways orchestrated by CD38 that are involved in myeloid maturation is important in understanding how ATRA works.

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 ATRA-induced differentiation, as evidenced by loss of nucleoskeleton remodeling and maturation markers [21-23]. These reports also show that ATRA 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 ATRA efficacy [14, 15]. 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.

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 ATRA-induced differentiation (Congleton et al., in press). 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 ATRA for 48 hours to induce CD38, and then treated with the SFK inhibitor PP2 for one hour

followed by IB4. We also used CD38<sup>+</sup> transfectants in these experiments to evaluate if there were signaling effects that were specific to ATRA or CD38 expression alone.

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 ATRA treatment [14, 15, 30, 43]. However it appears that CD38-propelled ERK phosphorylation is not mediated by Lyn kinase activity since PP2 failed to affect MAPK signaling in ATRA-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/ATRA co-treatment for 48 hours enhances Lyn expression along with Lyn/c-Raf and c-Raf/ERK interaction, and c-Raf C terminal domain (CTD) phosphorylation. These events may be facilitated by Lyn (Congleton et al., in press [44, 45]). 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 ATRA- and CD38-driven c-Cbl and p55/p85 PI3K phosphorylation, showing that Lyn regulates these events in both ATRA-treated HL-60 cells and CD38<sup>+</sup> transfectants. It is interesting that co-administration of ATRA 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 ATRA, blocked c-Cbl phosphorylation and impeded pY-p85 PI3K. Therefore Lyn kinase activity regulates CD38-stimulated signaling and ATRA-induced phosphorylation events that may be driven by CD38.

We also show that the loss of Lyn kinase activity coincided with a loss in interaction among CD38/SLP-76/Lyn, CD38/Vav1/Lyn, and CD38/Lyn/p85 PI3K. This suggests that the assembly of these CD38-associated complexes, which are likely involved in CD38 effector signaling after ATRA 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 ATRA, which significantly enhances differentiation, preserves Lyn kinase activity (Congleton et al., in press). The mechanism by which ATRA protects Lyn from PP2 inhibition is not known. In contrast dasatinib is able to inhibit Lyn in the presence of ATRA and also abrogates c-Cbl tyrosine phosphorylation and significantly decreases p85 PI3K subunit activity driven by ATRA. Dasatinib still enhances differentiation with ATRA co-treatment, but to a significantly lesser extent than PP2. This suggests signaling that characterizes normal ATRA 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 summary CD38-propelled p85 PI3K and c-Cbl phosphorylation, which is characteristic of ATRA-induced differentiation, is mediated by Lyn kinase activity. To our knowledge this is the first report that Lyn regulates ATRA- and CD38-transduced signaling in myeloid leukemia cells, and elucidates how CD38 partner molecules including SLP-76 and Vav1 may regulate differentiation during ATRA or ATRA/PP2 co-treatment.

A special thanks to Dr. Miaoqing Shen for providing the data for the FRET experiments.

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## CHAPTER VI

### **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**

#### **6.1 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<sup>phox</sup>. 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.

## 6.2 Introduction

The Src family of tyrosine kinases (SFKs) are a unique group of enzymes that have diverse functions in cell proliferation, survival, differentiation, adhesion, and migration. They play important regulatory roles in hematopoiesis, but also contribute to hematopoietic cancers. One historically prominent paradigm of SFK action is positive regulation of MAPK signaling and cell proliferation, and contribution to cell transformation [reviewed in [1]].

SFK hyperactivity is commonly associated with acute and chronic myeloid malignancies. The proliferative signals resulting from the BCR/ABL fusion tyrosine kinase in chronic myelogenous leukemia (CML) are driven by downstream SFKs including Src, Lyn, and Hck [2, 3]. Lyn is the predominant active SFK expressed in AML cells [4, 5]. It is often hyperactivated, is associated with iminitab resistance in CML, and may mediate the effects of the FLT3/ITD mutation found in 30% of AML cases [6-9]. Blocking SFK activity has been effective in slowing leukemic cell growth [10]. The inhibitor dasatinib has proven clinically successful in the treatment of CML, Philadelphia chromosome-positive acute lymphocytic leukemia (ALL) [11], and iminitab-resistant leukemias [12-14].

SFK activity and expression could also modulate ATRA differentiation induction therapy. Miranda et al. recently reported that the SFK inhibitor PP2 potentiated ATRA-induced gene expression and enhanced the differentiation marker CD11b in myeloid NB4, HL-60, and primary acute promyelocytic leukemia (APL) cells [15]. Kropf et al. recently reported that dasatinib also increased ATRA-induced CD11b expression [5]. In contrast, some reports show that SFKs may positively regulate ATRA-induced differentiation. Lyn and Fgr are upregulated in HL-60 and

NB4 myeloid leukemia cells after ATRA treatment, and both were reported to prevent apoptosis during granulocytic differentiation [16, 17].

SFK inhibitors are capable of positive and negative regulatory effects on MAPK pathway components. PP2 enhances Ras-independent Raf-1 activation that is mediated by Raf S621 phosphorylation [18], suggesting that SFK inhibitors are able to positively regulate Raf activity. Dasatinib, however, inhibits MAPK activity in the absence of growth factors (GFs) and attenuates signaling in the presence of GFs in CML progenitors [19]. MAPK augmentation may have implications for ATRA induction therapy, since retinoic acid results in sustained MAPK activity which is characteristic of HL-60 maturation [20-22].

The ability of SFKs to regulate ATRA-induced differentiation and MAPK signaling is therefore not understood. This motivates interest in how SFK inhibitors can affect the extent of ATRA-induced phenotypic conversion or modulate MAPK regulatory molecules. While ATRA is proven to be an effective intervention modality for t(15,17) positive APLs, it has not been effective in other leukemia subtypes, making means of improving its action in t(15,17) negative cells of therapeutic interest.

In this report the extent to which SFK inhibitors affect differentiation, myeloid leukemia cell phenotypic conversion, and MAPK signaling was characterized in t(15,17) negative HL-60 and t(15,17) positive NB4 cells. We specifically analyzed the effects of PP2 and dasatinib on two ATRA-regulated SFK members, Fgr and Lyn [16, 23]. While Fgr activation was undetectable in HL-60 cells, we found that the inhibitors had different effects on Lyn active site phosphorylation and cellular tyrosine phosphorylation in ATRA-treated cells. Both, however, were able to enhance the ATRA-induced phenotypic conversion and cell cycle arrest in two cell lines. Both

inhibitors also increased expression of Lyn and c-Raf, along with their interaction.

Phosphorylation of c-Raf at S259 (c-RafpS259) and C-terminal serine residues was increased, as well as c-RafpS259 and Lyn association. Lyn knockdown prevented ATRA-induced c-RafpS259 and CTD phosphorylation. CK2 co-immunoprecipitated with c-RafpS259, possibly modulating phosphorylation. ERK, which is also capable of phosphorylating Raf, showed increased interaction with c-Raf suggesting a MAPK feedback module consistent with the observed increase in C-terminal serine phosphorylation. These activities appear to be associated with the KSR1 scaffold protein. Similar results were observed for HL-60 and NB4 cells, indicating that combination inhibitor/ATRA therapy may be effective in a variety of myeloid leukemia cell types. Our results suggest a previously unreported MAPK-linked mechanism associated with accelerated ATRA/SFK inhibitor combination therapy.

### **6.3 Materials and Methods**

*Cell culture.* HL-60 and NB4 cells were grown in RPMI 1640 with 1% antibiotic/antimycotic from Invitrogen (Carlsbad, CA) and treated with ATRA as previously described [24]. PP2 and PP3 from EMD Chemicals (Gibbstown, NJ) were solubilized in dimethyl sulfoxide (DMSO) at 10 mM. Cells were treated with a final concentration of 10  $\mu$ M 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 hemacytometer and trypan blue exclusion.

*Antibodies and reagents.* Antibodies for cytometric analysis of CD11b and for CK2 Western

blotting were from BD Pharmingen (San Jose, CA). Protein A/G beads used for immunoprecipitation and p-Tyr antibody were from Santa Cruz Biotechnology. GAPDH, p-RafS259, p-MEK, p-Erk1/2, ERK1/2 (rabbit), KSR1, c-Raf (rabbit), pan-SFK Y416, Lyn, Fgr, HRP anti-mouse, and HRP anti-rabbit were from Cell Signaling (Danvers, MA). p-RafS621, c-Raf (mouse), and Lipofectamine 2000 CD were from Invitrogen. ERK1/2 (mouse) was from AbCam (Cambridge, MA). 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.

*Flow cytometric phenotypic analysis.* Immunostaining for CD11b was performed as previously described [20] and fluorescence was detected using a Becton Dickinson LSR II flow cytometer (San Jose, CA). Cell cycle analysis was performed as previously described [20].

*Western blot analysis and immunoprecipitation.* For immunoprecipitation experiments, cells were lysed as previously described [24]. Equal amounts of protein were pre-cleared with Protein A/G beads. The beads were pelleted and supernatant was incubated with appropriate antibodies and 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 [24].

*Creation of shLyn stable transfectants.* Plasmid containing shRNA targeting Lyn (target sequence: cgagcggagaactacatt) was from GeneCopia (Rockville, MD). 50µg of plasmid and 50µL of Lipofectamine 2000 CD (Invitrogen) were incubated at 25°C for 25 minutes in serum-free RPMI 1640 media.  $20 \times 10^6$  HL-60 cells were pelleted by centrifugation and resuspended in media containing the Lipofectamine/DNA complexes. The cells were electroporated at

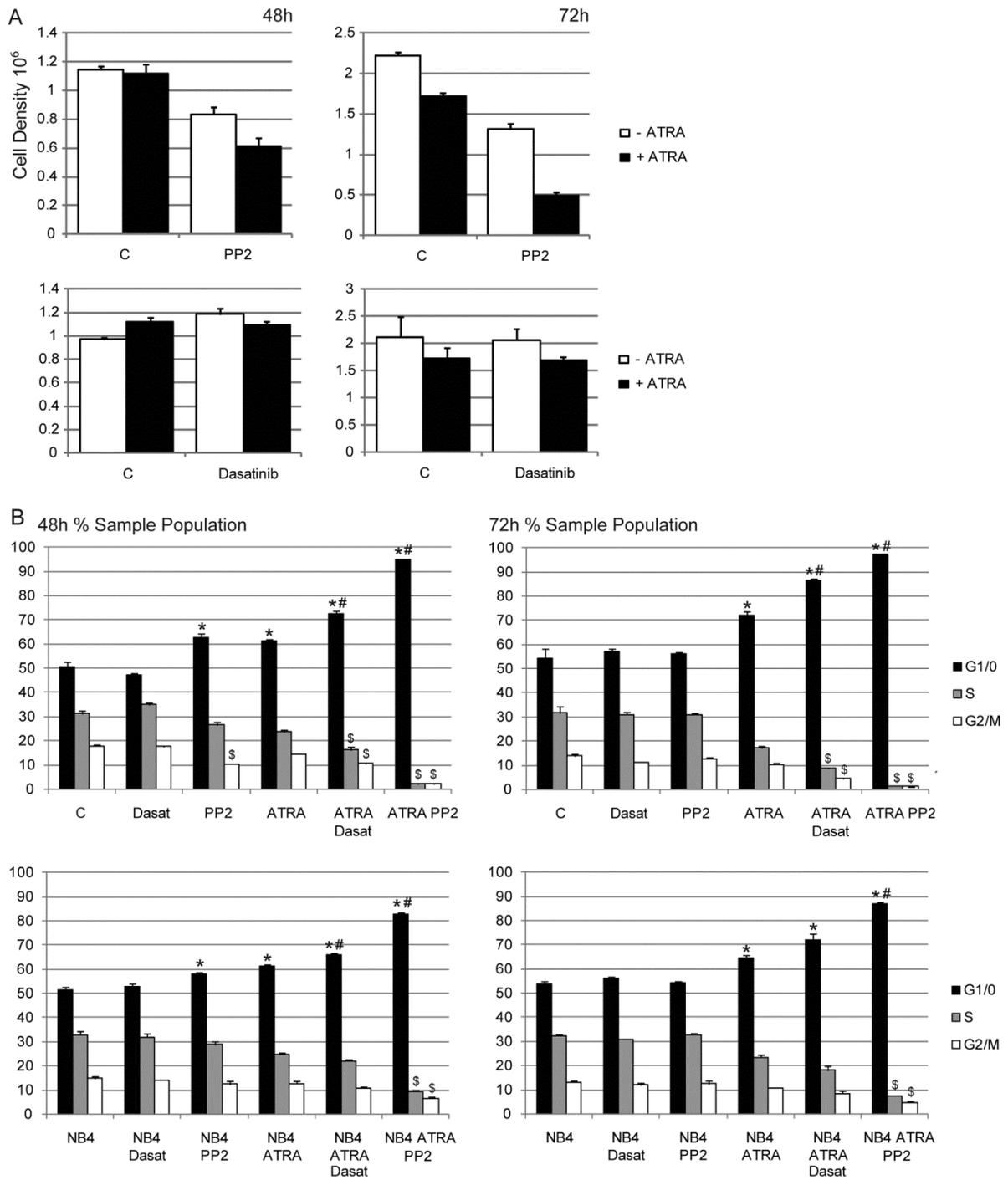
300mV/960 $\mu$ FD capacitance and immediately transferred to prewarmed media containing 10% FBS. After 24 hours debris was removed by centrifugation and cells were cultured as described. Cells expressing shRNA targeting Lyn were isolated by FACS sorting for eGFP.

*Statistics.* Statistics were analyzed using Microsoft Excel statistical software.

## 6.4 Results

*6.4.1 PP2 and dasatinib accelerate ATRA-induced G1/0 arrest.* To determine if PP2 and dasatinib affected ATRA-induced growth inhibition, we compared population growth during different treatment conditions in HL-60 cells. A DMSO vehicle control and PP3, an inactive structural analog of PP2, were included to test for toxicity or non-specific effects. These were used separately and in lieu of PP2 and had no significant effect on cell growth alone or in combination with ATRA (supplementary data S1a). No differences were detected among treatment groups after 24 hours. After 48 hours of culture PP2 alone decreased proliferation, and cells treated with a combination of PP2 and ATRA show greater growth inhibition (Figure 6.1a, top). Strikingly, PP2/ATRA co-treated cells showed no proliferation after 48 hours. The cells appeared to have undergone only one doubling followed by G1/0 arrest, consistent with a G1 cell cycle block. Unlike PP2, dasatinib had no significant effect on cell population growth (Figure 6.1a, bottom).

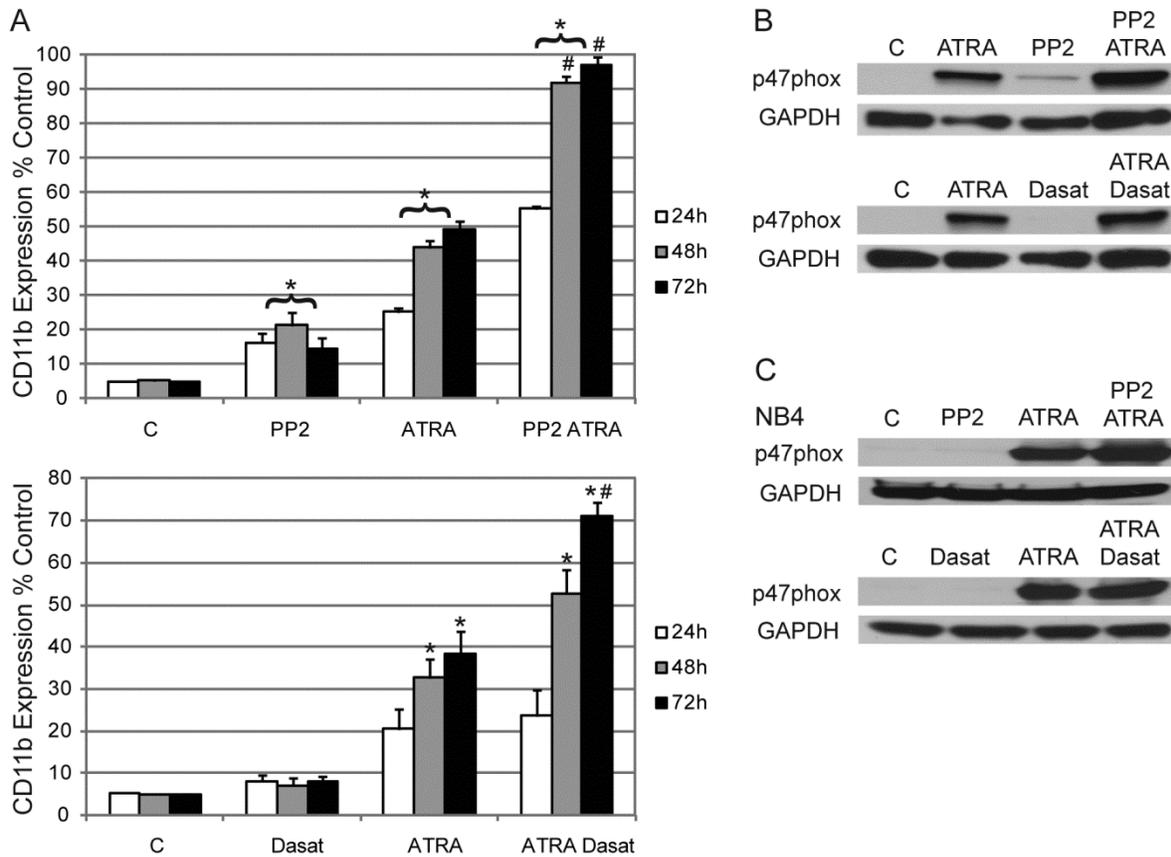
Next we measured cell cycle phase distribution in HL-60 and NB4 cells. After 48 and 72 hours all ATRA-treated and ATRA plus PP2 or dasatinib co-treated populations showed significantly more G1/0 compared to untreated cells or cells treated with dasatinib only (Figure 6.1b). PP2-treated populations showed a modest G1/0 enrichment in both cell lines at 48 hours. We also



**Figure 6.1 PP2 and dastinib enhance ATRA-induced growth arrest.** A: HL-60 cell counts at various time points using a hemacytomter. Trypan blue exclusion staining was used to evaluate apoptosis. B: Cell cycle analysis of HL-60 and NB4 cells with flow cytometry (\* $p < 0.5$  significantly higher than untreated control; # $p < 0.5$  significantly higher than ATRA treatment only; \$ $p < 0.5$  significantly lower than ATRA treatment only.)

detected a corresponding decrease in S and G2/M phase at 72 hours in all ATRA plus PP2 co-treated samples. HL-60 cells also showed a significant decrease in G2/M within 48 hours with co-treatments, and NB4 cells with ATRA plus PP2. Strikingly, approximately 95% of HL-60 cells and 85% of NB4 cells that received PP2/ATRA co-treatment were arrested in G1/0 by 48 hours. Although PP3 or DMSO in combination with ATRA showed slight albeit statistically significant G1/0 enrichment in some HL-60 sample sets, these increases were remarkably less than PP2 plus ATRA (Figure S1b). Therefore, the effects of PP2 on cell cycle and growth are largely attributable to SFK inhibition. Together these results suggest that both inhibitors propel ATRA-induced growth arrest, but PP2 appears more effective. Trypan blue exclusion staining and cytometric analysis of sub-G1 populations indicated that cells were arrested without evidence of apoptosis.

*6.4.2 PP2 and dasatinib modulate expression of ATRA-induced differentiation markers.* To determine if PP2 or dasatinib could further enhance ATRA-induced phenotypic conversion we evaluated the expression of myeloid differentiation markers. The  $\alpha$ -integrin receptor CD11b is upregulated after ATRA treatment and is considered a traditional marker of myeloid differentiation. PP2 and dasatinib increased ATRA-induced CD11b expression in HL-60 cells by 48 and 72 hours (Figure 6.2a). PP2 showed a more dramatic enhancement and had significant effects within 24 hours. This is consistent with previous reports [5, 15]. PP3 also enhanced ATRA-induced and CD11b expression at some time points, but only modestly compared to PP2 (Figure S1c). Interestingly, within 24 hours PP2 alone caused a modest induction of CD11b expression (Figure 6.2a), indicating that PP2 could propel at least some differentiation. Dasatinib alone had no effect on CD11b expression. The leukocyte antigen and differentiation marker



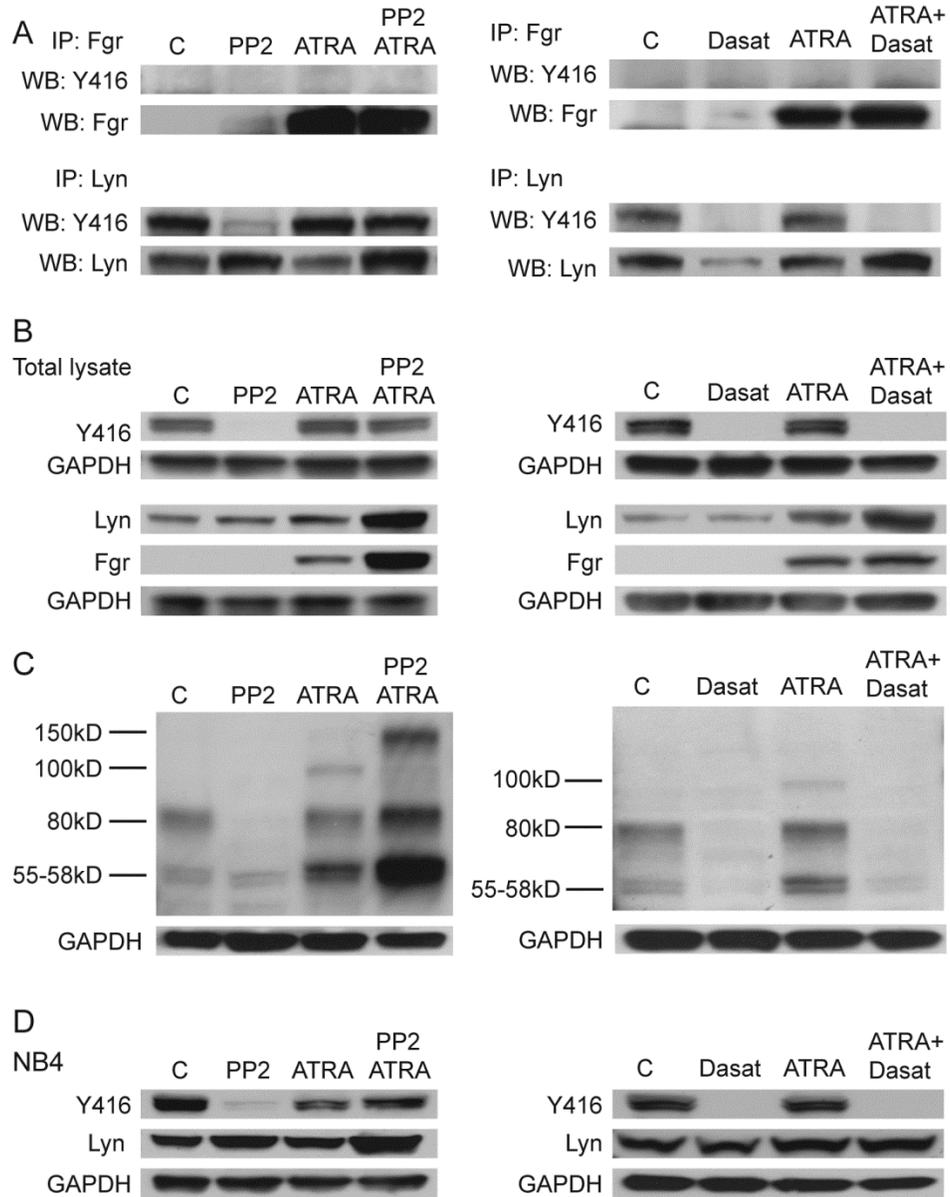
**Figure 6.2 SFK inhibitors enhance the expression of differentiation markers.** A: HL-60 cells were treated as shown and immunostained for CD11b at indicated time points. Gating was set to exclude 95% of the negative control (untreated cells). (\* $p < 0.5$  higher than untreated cells; # $p < 0.5$  higher than cells treated with ATRA only.) B: Western blots for p47<sup>phox</sup> expression in HL-60 cells after 48 hours of culture. C: Western blots for p47<sup>phox</sup> expression in NB4 cells after 48 hours of culture. GAPDH was used as a loading control for B and C.

CD38 was also evaluated but neither PP2 nor dasatinib had any significant effect on expression (data not shown).

Maturing myeloid cells are capable of induced respiratory burst/oxidative metabolism by an activated NADPH oxidase complex. p47<sup>phox</sup>, an NADPH oxidase component, is upregulated after ATRA. Within 48 hours of culture, cells treated with ATRA alone show enhanced expression of p47<sup>phox</sup> compared to the untreated control in NB4 and HL-60 cells (Figures 6.2b&c). PP2 alone modestly upregulated p47<sup>phox</sup> in HL-60 cells supporting evidence that it may induce differentiation. Co-treatment with either of the inhibitors modestly enhanced ATRA-induced p47<sup>phox</sup> expression in both cell lines. Together these results indicate that both inhibitors enhanced ATRA-induced differentiation markers.

*6.4.3 PP2 and dasatinib enhance ATRA-induced Fgr and Lyn expression, but differentially affect Lyn activation.* After ATRA treatment HL-60 cells show upregulated expression and tyrosine phosphorylation of Fgr and Lyn, while siRNA targeting Fgr or Lyn promotes apoptosis [16]. Since these ATRA-regulated SFKs may participate in induced differentiation, we determined the effects of the inhibitors by comparing protein expression and active site phosphorylation with different treatments.

Fgr was not detectable in HL-60 cells until they were treated with ATRA, which resulted in clear induced expression (Figures 6.3a&b). Co-treatment with ATRA plus either inhibitor enhanced Fgr expression (Figure 6.3b). Lyn was also upregulated by ATRA, and expression was further enhanced by co-treatment with PP2 or dasatinib. Therefore, ATRA/SFK inhibitor co-treatment increased protein expression of ATRA-regulated SFKs. Using a pan-SFK antibody that detects active site phosphorylation (Y416) in all family members, we found that activation in



**Figure 6.3 Inhibitors differentially affect Lyn activity but upregulate total Fgr and Lyn expression.** A: HL-60 cells were treated as indicated for 48 hours followed by immunoprecipitation (IP) or Western Blotting (WB). Lyn and Fgr IP membranes were probed using a pan-SFK Y416 antibody that detects tyrosine phosphorylation at the active site for all SFK members. Membranes were stripped and reprobed for total Lyn and Fgr protein. B: HL-60 Western blots for phosphorylated SFK members (Y416) and total protein for Lyn and Fgr. C: Total cell lysate was Western blotted for tyrosine phosphorylated proteins using a p-Tyr antibody. D: NB4 cell Western blots for active and total Lyn expression.

immunoprecipitated Fgr (FgrY412) was undetectable regardless of treatment, indicating its kinase function is not involved (Figure 6.3a, top). This suggested that a previous report [16] of upregulated Fgr tyrosine phosphorylation after ATRA may be relevant to the C-terminal autoinhibitory site.

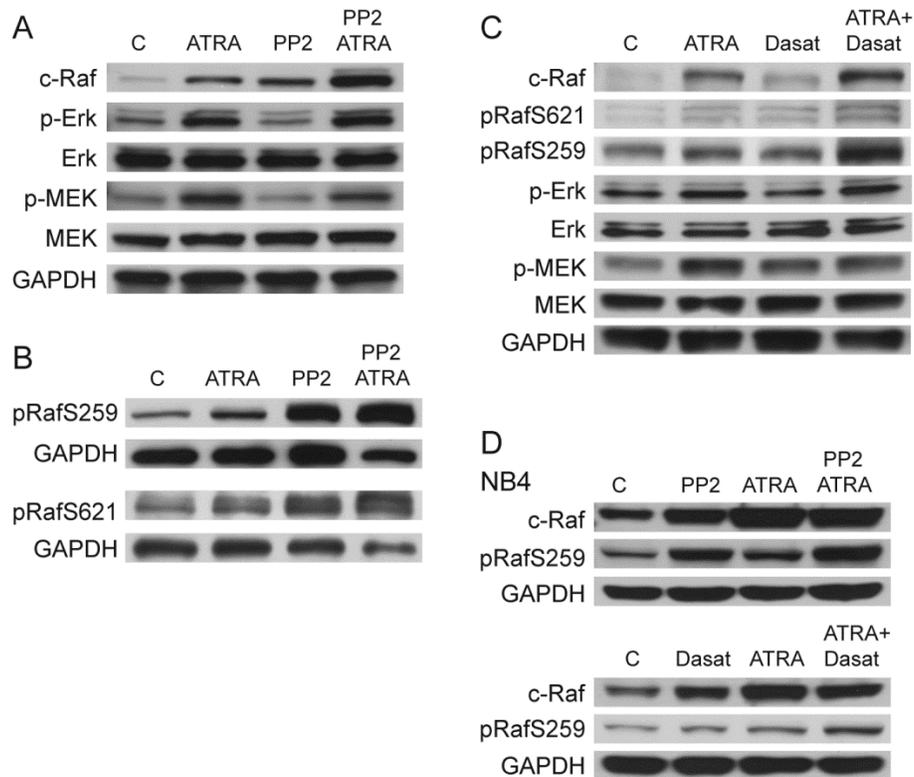
Evaluation of immunoprecipitated Lyn showed that PP2 and dasatinib crippled basal Y416 phosphorylation (LynY397) (Figure 6.3a, bottom). However in ATRA-treated cells, dasatinib continued to extinguish Lyn active site phosphorylation while PP2 did not. ATRA apparently protected Lyn from PP2 in this regard. Consistent with these results pan-SFK Y416 Western blotting showed that PP2 and dasatinib both abolished basal SFK activation, but while dasatinib continued to block SFK activity after ATRA treatment PP2 failed to do so (Figure 6.3b). Thus, it seems Lyn is the dominant active SFK in HL-60 myeloid leukemia cells consistent with a previous report [5]. This suggests that Lyn activity is dispensable for ATRA induction since cells co-treated with either inhibitor showed enhanced growth arrest and differentiation marker expression.

p-Tyr Western blotting showed that while PP2 alone decreased cellular tyrosine phosphorylation, it enhanced the p-Tyr status of at least two ATRA-modulated proteins in the range of 55-58 KD and 80 kD, upregulated the phosphorylation of a protein at approximately 150kD, and inhibited a 100kD protein (Figure 6.3c). In contrast, dasatinib appeared to extensively block cellular tyrosine phosphorylation alone and in combination with ATRA. This supports the argument that Lyn expression and activity plays a role in mediating ATRA-induced protein tyrosine phosphorylation.

Finally, we evaluated the effects of PP2 and dasatinib on Y416 phosphorylation and Lyn expression in NB4 cells (Figure 6.3d). Like HL-60 cells, we found that PP2 failed to block Y416 phosphorylation in the presence of ATRA while dasatinib was effective. PP2 alone and with ATRA increased total Lyn expression; this increase was enhanced by co-treatment. Dasatinib alone did not increase Lyn expression but ATRA and co-treatment did. Therefore, treatment of NB4 and HL-60 cells with either inhibitor plus ATRA upregulated Lyn expression, coinciding with enhanced differentiation.

*6.4.4 PP2 and dasatinib upregulate ATRA-induced c-Raf phosphorylation without affecting ERK or MEK activation.* ATRA-induced differentiation is driven by a sustained MAP kinase signal such that inhibition of MEK activity blocks ERK and c-Raf activation, preventing differentiation [21, 22]. Because SFKs can regulate MAPK signaling we determined the effect of PP2 and dasatinib on the Raf/MEK/ERK axis.

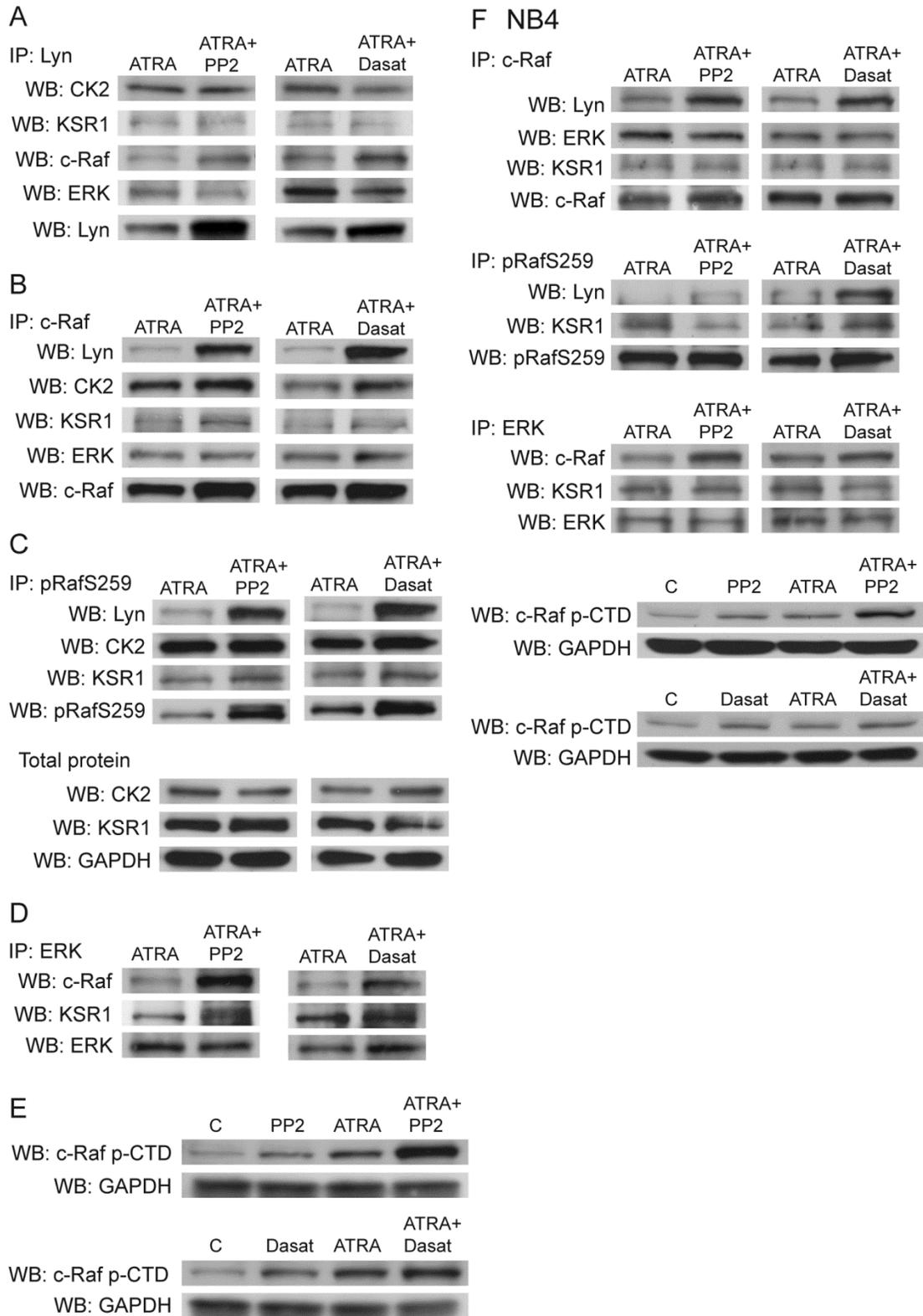
Like ATRA, PP2 alone upregulated c-Raf expression in HL-60 cells, and co-treatment showed further enhancement (Figure 6.4a). PP2 did not affect ATRA-induced phosphorylation of ERK or MEK or total ERK or MEK expression. We then evaluated the phosphorylation status of c-Raf regulatory residues. ATRA induces c-Raf S621 phosphorylation by 48 hours and coincides with nuclear migration [25], and ectopic expression of the Raf CR3 domain containing S621 enhances ATRA-induced differentiation [26]. PP2 alone and with ATRA increased c-Raf phosphorylation at S621 within 48 hours and also upregulated c-RafpS259 phosphorylation (Figure 6.4b). PP2 by itself and with ATRA strongly enhanced S259 phosphorylation. To our knowledge this is the first report of c-RafpS259 modification after ATRA or SFK inhibitor treatment.



**Figure 6.4 SFK inhibitors modulate c-Raf but not MEK and ERK.** A: HL-60 cells were treated as indicated for 48 hours. Western blotting shows phospho- and total ERK and MEK, and total c-Raf. B: HL-60 Western blots for phosphorylated c-RafS259 and S621 after 48 hours of culture. C: Western blots for MAPK signaling molecules and their phosphorylation status in HL-60 cells after 48 hours of treatment as indicated. D: Phosphorylation status of c-Raf in NB4 cells after 48 hours of treatment as indicated. GAPDH was used as a loading control for all blots.

We then determined if dasatinib had similar effects. Dasatinib alone showed little enhancement of c-Raf expression or phosphorylation of c-Raf at S621 and S259 (Figure 6.4c). However, co-treatment increased expression and phosphorylation at S621, with the most significant increase in c-RafpS259. Like PP2 dasatinib did not cause detectable differences in MEK or ERK expression or phosphorylation after ATRA. This suggests that PP2 and dasatinib have similar effects on the Raf/MEK/ERK axis after ATRA treatment. Consistent with this, experiments with NB4 cells showed an increase in c-RafpS259 and c-Raf expression after ATRA or inhibitor treatment (Figure 6.4d). c-RafpS259 also increased with ATRA or PP2 (but not dasatinib alone), and combination treatment with either inhibitor plus ATRA further enhanced phosphorylation. These results focused attention on c-Raf as a downstream target of PP2 and dasatinib. This motivated interest in identifying c-Raf and c-RafpS259 partners, particularly if there were associations between c-Raf and Lyn or other MAPK signaling regulators.

*6.4.5 PP2 and dasatinib regulate interactions between Lyn/c-Raf and ERK/c-Raf, and c-Raf phosphorylation.* Since the above results suggest a linkage between Lyn and c-Raf, there was interest in exploring associations between c-RafpS259 and Lyn, and also with CK2 and KSR1, two Raf regulating kinases [27-30]. We first evaluated if the inhibitors affected MAPK signaling molecule associations in HL-60 cells. Lyn and c-Raf immunoprecipitation experiments showed that these two molecules complex with each other after ATRA treatment, and that this interaction is increased by the addition of either PP2 or dasatinib (Figure 6.5a&b). Immunoprecipitated Fgr did not show significant interaction with c-Raf (data not shown).



**Figure 6.5 SFK inhibitors regulate interactions between Lyn and MAPK signaling molecules.** A: After 48 hours, Lyn was immunoprecipitated from HL-60 samples treated as shown. Western blotting shows interaction with partner molecules and Lyn confirms the IP. B: After 48 hours, c-Raf was immunoprecipitated from HL-60 samples treated as shown. Western blotting shows interaction with partner molecules and c-Raf confirms the IP. Interaction with Lyn and Lyn-complexed proteins detected in (A) was confirmed. C: After 48 hours, c-RafpS259 was immunoprecipitated from HL-60 samples treated as shown. Protein interaction with Lyn, CK2, and KSR1 was confirmed. Western blotting for CK2 and KSR1 show total protein expression. D: ERK was immunoprecipitated from HL-60 samples after 48 hours, and membranes were probed to confirm interaction with c-Raf and KSR1, suggesting a KSR1 scaffolded ERK-mediated feedback loop. E: HL-60 cell Western blot for c-Raf CTD phosphorylation of either S289, S296, or S301. F: Immunoprecipitation experiments with NB4 cells confirm increased interaction with Lyn/c-Raf, Lyn/c-RafpS259, c-Raf/ERK, and potential KSR1 scaffolding. Western blotting confirms increases in CTD phosphorylation with ATRA/inhibitor co-treatment.

The serine/threonine (S/T) kinase, casein kinase II (CK2), is known to complex with and be phosphorylated by Lyn and Fgr [31, 32]. CK2 also interacts with KSR1, a scaffold protein that modulates MAPK signaling [[30] and reviewed in [33]]. CK2/KSR1 binding facilitates Raf phosphorylation, which is dependent on SFK-mediated Raf Y341 phosphorylation [28].

Therefore, Lyn may be linked to MAPK signaling and c-Raf binding through interactions with CK2 and KSR1. We immunoprecipitated Lyn and c-Raf and found that CK2 and KSR1 complex with c-Raf and Lyn (Figure 6.5a&b). We also detected ERK binding to Lyn and c-Raf, which is also reported to interact with KSR1 [30, 34]. These results raised the possibility that Lyn/c-Raf binding may facilitate a CK2/c-Raf complex that takes place on a KSR1 scaffold, which may include ERK. MEK also precipitated with c-Raf and Lyn in ATRA-treated cells but the amount of interaction was unaffected by the inhibitors (data not shown).

Since CK2 is an S/T kinase and we observed a significant increase in c-Raf phosphorylation at S259, we precipitated c-Raf<sup>S259</sup> and probed for Lyn, CK2, and KSR1 (Figure 6.5c). Co-treatment with either inhibitor plus ATRA increased interaction between c-Raf<sup>S259</sup> and Lyn. We were also able to detect CK2 and KSR1 interaction with pRaf<sup>259</sup>, consistent with these proteins existing in a KSR1-scaffolded complex. Total protein expression of CK2 and KSR1 did not change after inhibitor co-treatment (Figure 6.5c).

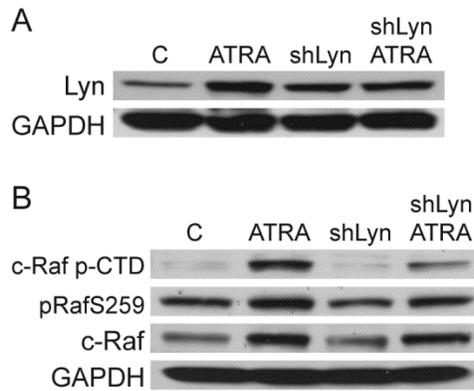
KSR1/ERK interactions can control MAPK signaling specificity and duration, as well as cell proliferation [30, 35, 36]. We found that the inhibitors caused a notable enhancement in ERK interaction with c-Raf in ATRA-treated cells (Figure 6.5d). We also observed KSR1/ERK interaction. Although reciprocal experiments with immunoprecipitated c-Raf did not show such a pronounced increase in ERK interaction, this could be attributed to differences in protein

abundance and different proportions of c-Raf or ERK protein that are heterodimerized with each other.

ERK participates in Raf feedback phosphorylation of serine residues in the C-terminal domain (CTD), which include S289, S296, and S301 [37, 38]. Therefore we investigated CTD phosphorylation status. We found that PP2 or dasatinib alone increased p-CTD, which was enhanced by ATRA and further increased by co-treatment (Figure 6.5e).

Immunoprecipitation experiments with NB4 cells were consistent with these results (Figure 6.5f). c-Raf and c-RafpS259 showed increased interaction with Lyn after ATRA/inhibitor co-treatment within the context of a potential KSR1 scaffold. Co-treatment also increased interaction between ERK and c-Raf. Finally, p-CTD was increased by ATRA combined with PP2 and to a lesser extent dasatinib, although these increases were not as striking compared to HL-60 cells. Together, these results suggest that the inhibitor-induced increase in c-Raf and Lyn expression facilitates an increase in their interaction, which is accompanied by CK2 and KSR1 binding. This may be consistent with CK2 kinase activity toward c-Raf that results in c-RafpS259 phosphorylation, and the ability of KSR1 to act as a scaffold. PP2 and dasatinib enhanced ERK association with c-Raf which is consistent with ERK feedback phosphorylation within the CTD, also in the context of the KSR1 scaffold.

*6.4.6 Lyn knockdown decreases c-RafpS259 and CTD phosphorylation.* To evaluate whether downregulating Lyn expression would interfere with ATRA-induced c-Raf phosphorylation, we created a stably transfected cell line expressing shRNA targeted against Lyn (shLyn). Lyn expression in untreated shLyn cells was similar to wild-type HL-60 cells, but ATRA could no longer upregulate Lyn (Figure 6.6a). After ATRA treatment, transfectants were still capable of



**Figure 6.6 Lyn knockdown decreases c-Raf phosphorylation.** A: HL-60 cells and shLyn stable transfectants were incubated with ATRA for 48 hours followed by Western blotting for total Lyn expression. B: Western blotting for c-Raf CTD serine phosphorylation, c-RafpS259, and total c-Raf after 48 hours of culture with or without ATRA. GAPDH was used as a loading control.

c-Raf upregulation but showed decreased phosphorylation of c-RafS259 and CTD serine residues (Figure 6.6b). Therefore, blocking ATRA-induced Lyn upregulation interfered with c-RafS259 and p-CTD that is characteristic of HL-60 myeloid differentiation. These results suggest that the ATRA-induced increases in Lyn expression modulates MAPK signaling through c-Raf.

We evaluated if Lyn knockdown affected G1/0 arrest or CD11b expression, but there were no significant differences between HL-60 cells and shLyn transfectants. This is likely a result of achieving only partial Lyn knockdown. As seen in Figure 6.6a, we were only able to decrease ATRA-inducible Lyn expression. It is possible that there was still enough Lyn protein in shLyn cells to permit cell cycle arrest and CD11b upregulation. The effects we did observe in the transfectants that were specific to c-Raf are likely most evident because Lyn has a specific role in increased phosphorylation of S259 and CTD residues.

## **6.5 Discussion**

ATRA has been successfully used to treat APL for many years. However, patients can develop resistance to treatment, and those presenting t(15,17) negative AML have not been responsive to ATRA therapy alone. Therefore alternative or combination therapies could improve prognosis and survival.

We found that co-treating t(15,17) negative HL-60 or t(15,17) positive NB4 cells with ATRA plus either dasatinib or PP2 (SHK inhibitors) caused significant G1/0 DNA enrichment within 48 hours compared to ATRA alone. The inhibitor-induced cell cycle arrest led to an investigation of differentiation marker effects. Both dasatinib and PP2 enhanced the ATRA-induced upregulation of the  $\alpha$ -integrin receptor CD11b and NADPH oxidase protein p47<sup>phox</sup>. PP2 by itself also

appeared able to induce some cell differentiation. These results show that SFK inhibitors increase cell cycle arrest and differentiation markers in ATRA-treated cells that are either t(15,17) positive or negative, suggesting that combination therapy may improve ATRA effectiveness in different types of leukemia.

We then investigated the effects of the inhibitors on Lyn and Fgr, which are upregulated and tyrosine phosphorylated after ATRA treatment [16, 23] and therefore may regulate differentiation. ATRA increased Lyn and Fgr expression in HL-60 cells, and co-treatment with either inhibitor plus ATRA further enhanced expression. NB4 cells also showed Lyn upregulation. Fgr active site phosphorylation was not detectable in any samples, suggesting that the previously reported increase in phosphorylation after ATRA [16] may be specific to the inhibitory C terminal region. Since Fgr activity seemed irrelevant to induced differentiation, we turned our attention toward Lyn.

Dasatinib inhibited Lyn phosphorylation alone and after ATRA treatment in HL-60 and NB4 cells, but while PP2 alone was inhibitory ATRA treatment protected Lyn activity in co-treated cells. It is noteworthy that Lyn is the dominant active SFK in HL-60 and NB4 myeloid leukemia cells [5], yet the failure of PP2 to inhibit Lyn after ATRA still coincided with accelerated G1/0 arrest and phenotypic conversion. The mechanism by which ATRA protects Lyn from inhibition remains unknown. Together, these results suggest that SFK kinase activity is not necessary for differentiation. One could speculate that SKFs such as Lyn provide scaffolding functions similar to ERK, which can act as a scaffold independent of its kinase activity [39].

ATRA induction is characterized by MAPK activation, and inhibitors of MEK and c-Raf block differentiation [21, 22]. Neither PP2 nor dasatinib affected ATRA-induced ERK or MEK

phosphorylation. However both inhibitors further enhanced ATRA-upregulated c-Raf expression in both cell lines. The increase in c-Raf is significant, since expression of activated c-Raf drives ATRA-induced differentiation [26]. Dasatinib and PP2 also enhanced ATRA-induced c-Raf phosphorylation at S621, which is associated with differentiation [25]. Most strikingly, co-treatment with ATRA alone and ATRA plus either inhibitor increased c-RafpS259 phosphorylation in NB4 and HL-60 cells, implicating a previously unreported role for c-RafpS259 in ATRA-induced differentiation.

Dual phosphorylation of c-Raf S621 and S259 is characteristic of quiescent cells and associated with 14-3-3 binding which can inactivate Raf [38, 40, 41]. Cells expressed mutated c-RafpS259A show increased proliferation [38], which supports the argument that Raf S259 phosphorylation may attenuate mitogenic signaling. Although we did not observe any significant interruptions in ERK and MEK activation, MAPK signaling depends on the finely-tuned orchestration of interactions with scaffolds, regulator molecules, and positive and negative feedback loops that include direct regulators such as MEK and ERK. This motivated interest in whether there was interaction between c-RafpS259, SFKs, and other MAPK signaling proteins.

KSR1 is a scaffolding molecule that modulates interactions between Raf, MEK, and ERK, and fine-tunes the specificity of MAPK signaling [30]. It also interacts with casein kinase II (CK2), an S/T kinase known to complex with and be phosphorylated by Lyn and Fgr [32]. CK2 phosphorylates KSR1 and is part of the scaffolding complex that regulates MAPK signaling. Specifically, c-Raf Y341 is phosphorylated by SFKs; a modification that is reported to be a prerequisite for CK2 c-Raf S338 phosphorylation and is dependent on KSR1 scaffolding [28]. Our results showed that Lyn was able to complex with CK2 and KSR1, providing a link to

MAPK signaling proteins. Consistent with this, ATRA plus inhibitor treatment increased interaction between c-Raf and Lyn in HL-60 and NB4 cells. c-Raf also co-immunoprecipitated CK2 and KSR1 in HL-60 cells, suggesting a Lyn-containing CK2/MAPK complex scaffolded by KSR1. A potential KSR1 scaffolding function was also detected in NB4 cells. c-Raf<sup>pS259</sup> also showed increased interaction with Lyn, CK2, and KSR1. One could speculate that c-Raf phosphorylation at S259 is a result of CK2 kinase activity facilitated by Lyn interaction. Alternatively, c-Raf/Lyn binding could localize CK2 to one of its targets, many of which include key cell cycle regulators [reviewed in [42]].

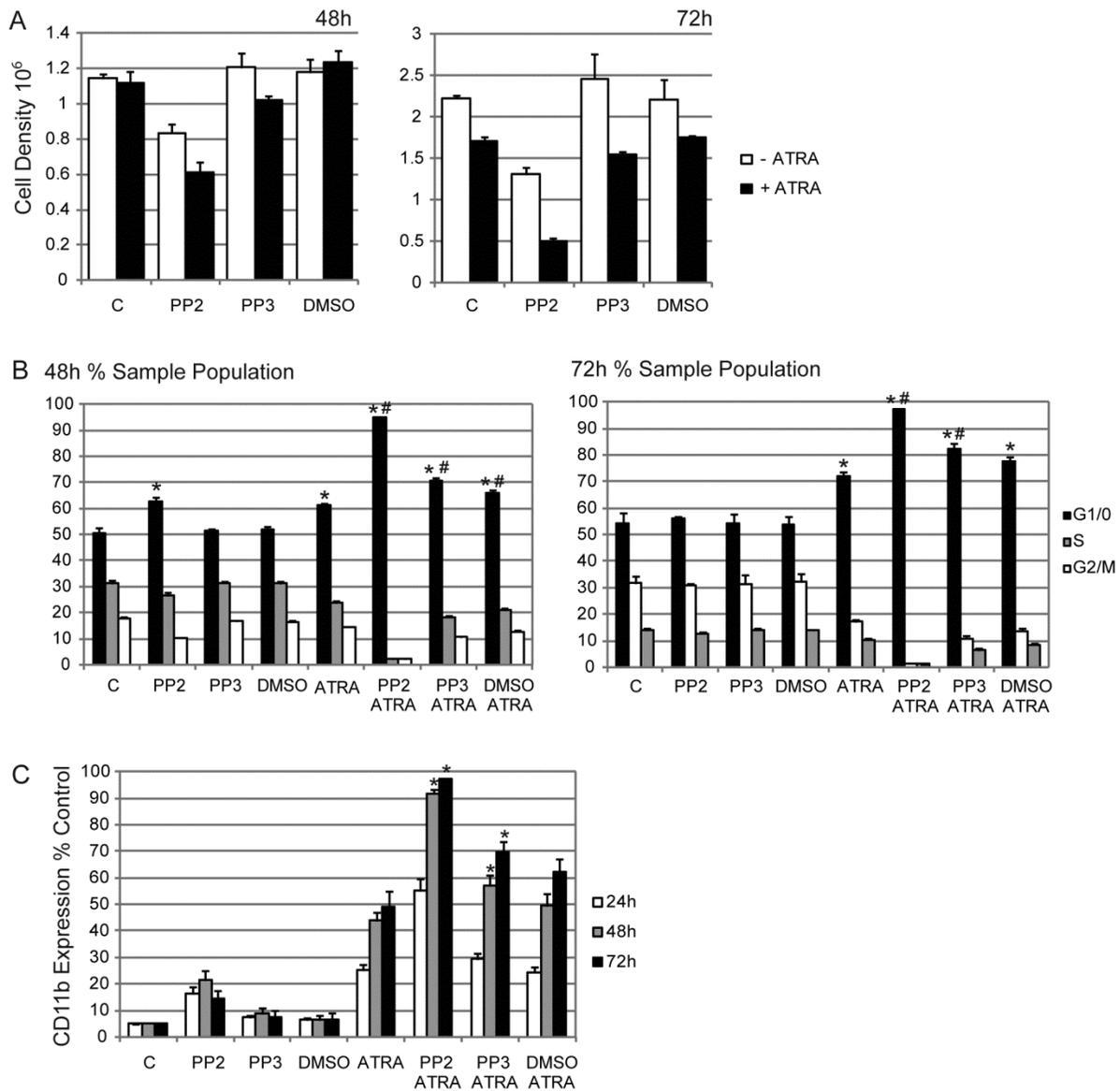
Consistent with a MAPK feedback mechanism, we found that after PP2 or dasatinib/ATRA co-treatment ERK showed more interaction with c-Raf, and also bound KSR1. This coincided with upregulated serine phosphorylation at the C terminus of c-Raf. ERK can directly mediate feedback phosphorylation of c-Raf on S287, S296, and S301, which controls Raf activation and modulates signaling [37, 38]. Thus co-treatment could be attenuating proliferative MAPK signaling through a KSR1 scaffolding complex containing a Lyn/CK2/c-Raf/ERK module.

Knockdown of Lyn by shRNA decreased ATRA-induced c-Raf<sup>pS259</sup> and CTD phosphorylation, suggesting that Lyn regulates c-Raf post-translational modifications. Therefore it is possible that alterations in c-Raf phosphorylation status and interactions facilitated by ATRA and Lyn are changing the character of the MAPK signaling cascade to regulate induced growth arrest and differentiation. Lyn-regulated MAPK orchestration through c-Raf modifications, specifically phosphorylation of S259 and the CTD, may reflect differential involvement of feedback loops with ERK that significantly slow proliferation and expedite cell cycle arrest and differentiation. For example, MAPK stimulation by different growth factors creates positive or negative

feedback loops that result in differentiation or proliferation respectively by fine-tuning magnitude and longevity [43, 44].

In conclusion, SFK inhibitors may have the potential to modulate MAPK signaling to enhance the therapeutic efficacy of ATRA in the treatment of a variety of myeloid leukemias. Co-treatment with ATRA plus PP2 or dasatinib may accelerate the phenotypic conversion of AML and APL cells to differentiating myeloid cells by targeting specific molecular markers that are characteristic of ATRA-induced differentiation. The identification of potential molecular targets and mechanisms that may improve the clinical benefit of ATRA encourages further evaluation of ATRA/SFK inhibitor combination therapy.

Supplementary Data 1



**Supplementary Data 1.** A: HL-60 cells were treated as indicated. Growth and viability were measured using a hemacytometer and Trypan Blue exclusion staining. B: HL-60 cells were treated as indicated and cell cycle phase was determined by propidium iodide staining and flow cytometry (\* $p < 0.5$  significantly higher than untreated control; # $p < 0.5$  significantly higher than samples treated with ATRA only). C: HL-60 cells were treated as shown and immunostained for CD11b. Gating was set to exclude 95% of untreated control cells (\* $p < 0.5$  significantly higher than cells treated with ATRA only at the same time point).

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## CHAPTER VII

### DISCUSSION AND FUTURE DIRECTIONS

All-*trans* retinoic acid (ATRA) is clinically used to treat t(15,17) positive APL, but its effectiveness has been limited to this leukemia subpopulation since it has shown little therapeutic success with other types of cancers. Identifying molecules and signaling pathways that confer ATRA responsiveness could elucidate mechanisms of action that can be manipulated to expand the clinical usefulness of retinoic acid. In addition, the discovery of compounds that enhance the effects of ATRA and that could be used in combination therapy may address several of ATRA's current limitations, including resistance, relapse and RAS. In this report we identify several signaling molecules and therapeutic compounds that provide insight to ATRA responsiveness and may ultimately improve its clinical usefulness.

The results presented in this dissertation build upon the current knowledge of the role of CD38 in cell signaling and its receptor activity, specifically in differentiating myeloid leukemia cells. The data also identifies the SFK Lyn as a key regulator of CD38 signaling and ATRA-induced differentiation, and suggests that SFK inhibitors may be used clinically to enhance the therapeutic benefits of ATRA in a variety of leukemias. We also show ATRA- and ATRA/inhibitor modulation of the c-Raf/MEK/ERK axis, which elucidates important mechanisms of action involved in leukemic cell differentiation and potential therapeutic targets.

The leukocyte antigen CD38 is a multifunctional receptor and ectoenzyme that is differentially expressed during various stages of lymphocyte and myeloid activation and maturation (reviewed in [1]). It is also present in a variety of other cell types and has various implications in disease

including CLL, HIV/AIDS, and diabetes. The role of CD38 in leukemia and myeloid differentiation is enigmatic. Some reports implicate CD38 in cell proliferation, since interaction with an agonist ligand stimulated growth in HL-60, NB4, and patient leukemia cells [2-4]. Expression of CD38 in HeLa cells reduces doubling time by shortening S phase [5]. These results support a role for CD38 as a pro-proliferative receptor which could explain its identification as a negative prognostic marker in CLL. However other studies show that CD38 is important for leukemic cell differentiation, since siRNA targeting CD38 cripples the effects of ATRA while overexpression drives it [6, 7].

Since there is evidence for a causal role for CD38 in ATRA-induced differentiation, we investigated which domains of CD38 are required. The CD38 enzymatic domain is extracellular and metabolizes NAD<sup>+</sup> and NAADP<sup>+</sup> to form a variety of products including cADPR or NAADP which are calcium mobilizing molecules [8-12]. CD38 catalytic activity depends on the E226 residue in the active pocket, and covalent modification with the inhibitors (F-araNAD or F-araNMN) blocks its function as an enzyme [12-14]. As a receptor, it can propel phosphorylation of ERK, c-Cbl, and PI3K, and regulate other signaling pathways such as cytokine secretion [15-23]. We found that overexpression of CD38 containing a point mutation (E226Q) which blocks enzymatic activity was able to propel ATRA induction similar to transfectants overexpressing wild-type CD38. In addition, HL-60 cells treated with the specific enzymatic inhibitors F-araNAD or F-araNMN did not show differences in induced cell cycle arrest and phenotypic conversion when compared to cells only treated with ATRA. This indicated that the enzymatic activity of CD38 is not necessary for differentiation.

In contrast, the receptor activity of CD38 that is associated with its membrane expression is important for myeloid maturation. We deleted the transmembrane proximal portion of the CD38

cytosolic tail (CD38  $\Delta$ 11-20), which resulted in the loss of CD38 membrane localization. ATRA-treated CD38  $\Delta$ 11-20 transfectants showed crippled G1/0 arrest, decreased expression of the  $\alpha$ -integrin receptor CD11b, and virtually no inducible oxidative metabolism, all of which are considered traditional differentiation markers. In addition, CD38  $\Delta$ 11-20 blocked the ATRA-induced expression of Vav1 and Fgr. These results suggested that the loss of CD38 cell surface expression coincided with the loss of its ability to facilitate membrane-associated signaling complexes, and that such complexes may include SFKs such as Fgr and the GEF Vav1. It also raised the possibility that CD38  $\Delta$ 11-20 may be sequestering or improperly interacting with cytosolic proteins that would normally be membrane-associated, which may contribute to the block in differentiation. Future research could investigate how CD38  $\Delta$ 11-20 is interfering with ATRA's effects by identifying interaction partners that would normally complex with wild-type CD38 but are now being modulated by the cytosolic mutant. This could explain in part why CD38  $\Delta$ 11-20 appears to act as a dominant negative since the wild-type, endogenous CD38 induced in CD38  $\Delta$ 11-20 transfectants after ATRA treatment was unable to rescue differentiation.

It is noteworthy that CD38  $\Delta$ 11-20 transfectants were still capable of propelling ERK phosphorylation and therefore modulated a component of MAPK signaling. This showed that p-ERK is not sufficient to drive granulocytic maturation. It is possible that CD38  $\Delta$ 11-20 is changing the character of the MAPK signal to interfere with differentiation. CD38 is capable of propelling different types of MAPK signaling and could engage in signal switching. For example, CD38 agonist ligand interaction produces a transient p-ERK signal that is typically associated with cell growth and proliferation. However CD38 overexpression drives a persistent ERK signal that is associated with differentiation. The mutation of CD38 domains that are

important for receptor signaling may significantly perturb the regulation of signaling pathways such as the MAPK cascade that could have significant cellular outcomes.

CD38  $\Delta$ 11-20 cells were also unable to upregulate Vav1 which can modulate the Raf/MEK/ERK axis and therefore further perturb MAPK signaling [24, 25]. The literature supporting an integral role for Vav1 in ATRA induction is significant, and failure to upregulate Vav1 may also have repercussions besides MAPK signaling. For example, Vav1 is thought to regulate PI3K activity in ATRA-treated HL-60 and NB4 cells, which results in nucleoskeleton remodeling [26-28]. These reports also show increased Vav1 tyrosine phosphorylation and nuclear accumulation after ATRA treatment, and that downmodulation of Vav1 by siRNA interferes with changes in nuclear morphology that are characteristic of granulocytic maturation. Consistent with this overexpression of wild-type Vav1 potentiated the effects of ATRA by accelerating changes in nuclear morphology and CD11b expression, which is transcriptionally regulated by Vav1 [26, 29]. The SFKs Fgr and Lyn are also upregulated after ATRA and may play functional roles in granulocytic differentiation [30-32]. Therefore, the inability of CD38  $\Delta$ 11-20 cells to upregulate Vav1 and SFKs combined with the potential for CD38  $\Delta$ 11-20 protein to interact with other signaling molecules may have crippled ATRA responsiveness on several levels.

We also report the interesting result that while CD38  $\Delta$ 11-20 cells are incapable of ATRA-induced granulocytic maturation, they show normal 1,25-dihydroxyvitamin D3 (D3)-induced monocytic differentiation. This suggests that wild-type CD38 is not required for maturation along the monocytic lineage, or that signaling proteins modulated by ATRA and regulated by wild-type CD38 are not important for D3 induction. Further analysis comparing how CD38 functions in differentiating granulocytic cells versus monocytic cells would elucidate why wild-

type protein expression is needed for ATRA induction but not D3, and how CD38  $\Delta$ 11-20 may impede the therapeutic benefits of ATRA but not D3.

The revelation that the receptor functions of CD38 are required for ATRA induction but the enzymatic activity is not caused us to focus our studies on the identification of CD38 interaction partners and how these molecules are involved in CD38-stimulated signaling. Previous research showed that CD38 interacts with the adaptor and E3 ubiquitin ligase c-Cbl [33, 34]. Both molecules drive MAPK signaling when overexpressed, and a c-Cbl TKB domain mutant (G306E) that does not bind CD38 shows crippled differentiation and fails to drive MAPK signaling [35, 36]. This suggests that CD38 and c-Cbl may cooperatively contribute to the persistent MAPK signal required for ATRA efficacy, as well as regulate other signaling cascades. Previous reports show that c-Cbl binds Vav1, and the adaptor molecule SLP-76 that cooperates with c-FMS to propel differentiation [34, 37]. Therefore it was plausible that CD38 also forms a complex with these c-Cbl-associated proteins. In support of this, the current results show that CD38  $\Delta$ 11-20 transfectants fail to upregulate Vav1 as well as the SFK Fgr. Therefore wild-type CD38 signaling may be mediated through SFKs, c-Cbl, Vav1, and SLP-76 in a putative signaling complex, and we investigated this possibility.

Indeed, we detected interaction between CD38, Vav1, SLP-76, and Lyn in ATRA-treated HL-60 cells and CD38<sup>+</sup> transfectants. We did not detect c-Cbl/Lyn interaction indicating that CD38 is able to facilitate the assembly of different cytosolic complexes that regulate signaling, or that potential c-Cbl/Lyn binding is labile. It is interesting that the c-Cbl G306E mutant that cripples MAPK signaling and differentiation fails to bind CD38, but does co-precipitate with Vav1 and SLP-76 [34]. Therefore it is plausible that a constitutive cytosolic complex containing c-Cbl/Vav1/SLP-76 requires CD38-facilitated membrane recruitment through the c-Cbl TKB

domain to promote signaling after ATRA treatment. Further research could investigate if the TKB mutant also interrupts CD38/Vav1/SLP-76 binding.

It is noteworthy that untreated CD38+ transfectants showed increased binding between SLP-76 and Vav1, indicating that CD38 expression alone facilitates complex assembly. CD38 protein in untreated transfectants also shows significant interaction with SLP-76, Vav1, and Lyn, again demonstrating that expression alone facilitates complex formation without ATRA. However, since CD38+ cells do not spontaneously differentiate, it is clear that ATRA-induced or activated accessory molecules are required for CD38 to propel differentiation.

The type and magnitude of CD38 signaling could also change depending on different protein-protein interactions. Since the CD38 cytosolic domain is so short (20 amino acids) homo- or heterodimerization and lateral associations with other membrane proteins could facilitate cytosolic platforms for signaling complex assembly. It would be interesting to investigate which types of signaling are driven by dimerization, lateral associations on the cell membrane, or cytosolic complexes such as the CD38/Vav1/SLP-76/Lyn module identified here.

We also found that Lyn kinase activity regulates certain aspects of CD38-driven signaling. Incubating ATRA-treated cells with the SKF inhibitor PP2 for one hour was able to block CD38-stimulated tyrosine phosphorylation of p85 PI3K and c-Cbl. However protecting Lyn kinase activity by 48 hour co-treatment with PP2 and ATRA preserved pY-p85 PI3K and pY-c-Cbl. The inhibitor dasatinib, which blocks Lyn kinase activity in the presence of ATRA, was also able to interfere with ATRA-induced c-Cbl and p85 PI3K phosphorylation which may be regulated by CD38. Therefore, Lyn activity plays an integral role in these ATRA- and CD38- propelled events.

Previous reports show that c-Cbl Y731 phosphorylation creates a PI3K docking site and may lead to subsequent ubiquitination or sequestration, decreasing cell proliferation [38, 39].

Therefore it is possible that CD38 ligand-induced c-Cbl phosphorylation, which is facilitated by Lyn, creates an SH2 binding site for p85 PI3K that results in the downregulation of proliferative activity. Cbl-b suppression of PI3K activity in leukemia cells enhances chemotherapy-induced apoptosis and enhances ERK activation [40]. This could be one mechanism by which CD38 cooperates with c-Cbl to orchestrate MAPK signaling. In contrast, other reports show that CD38-mediated growth suppression requires PI3K activity and its association with c-Cbl [17, 18], which could require Lyn. A CD38/PI3K signaling axis could also be biphasic and switch character depending on a changing cellular landscape in response to ATRA treatment. For example, PI3K-induced Akt activation increases within the first 48 hours of ATRA treatment but then decreases by 72 hours [41].

Localization of p85 PI3K may be important as well, since several reports show PI3K activity facilitates nucleoskeleton remodeling and is required for ATRA-induced differentiation [27, 41, 42]. It is possible that c-Cbl sequesters PI3K in the cytosol while an active, unsequestered PI3K cooperates with nuclear Vav1 to facilitate structural changes in the nucleus. Future research is required to elucidate how the interaction, localization and post-translational modification of c-Cbl and p85 PI3K may regulate the effects of ATRA.

These studies also revealed that inhibition of Lyn activity decreases interactions between Vav1/CD38/Lyn, and SLP-76/CD38/Lyn. This suggests that phosphorylation events downstream of CD38 such as pY-c-Cbl and pY-p85 PI3K are propelled by a putative CD38/Vav1/SLP-76/Lyn signaling module. As described previously, Vav1 appears important for several GEF-independent aspects of induced differentiation. Therefore, it is plausible that Vav1 serves as an

adaptor or scaffold to facilitate or stabilize protein-protein interactions. For example, Vav1 was recently reported to act as a scaffold that stabilizes SLP-76 signaling microclusters [43].

We also detected a decrease in CD38/p85 PI3K interaction when Lyn was inhibited, and virtually no Lyn/p85 PI3K interaction. This suggested that Lyn kinase activity is required for its interaction with p85 PI3K and indicates a direct role in p85 regulatory subunit phosphorylation, consistent with the ability of SFKs to activate p85 PI3K (reviewed in [44]).

It is interesting that we did not detect any changes in CD38-stimulated ERK phosphorylation with Lyn inhibition, showing that CD38 differentially regulates signaling. This is consistent with CD38's ability to drive diverse cellular outcomes such as proliferation or differentiation. It is likely that different CD38-propelled signals are fine-tuned or switched by interactions with upstream and downstream regulators, scaffolds, and feedback loops. This is similar to MAPK signal orchestration which is dynamic and involves direct and indirect effectors that require scaffolding and feedback loops for fine-tuning and signal-switching (reviewed in [45]). An example of this was evidenced in the results described here using SFK inhibitors with ATRA co-treatment, where we detected a possible positive feedback mechanism involving c-Raf phosphorylation that enhanced differentiation.

In these studies, our discovery that Lyn regulates CD38 receptor signaling led us to further investigate the phenotypic effects of the SFK inhibitors PP2 and dasatinib on differentiation induced by ATRA. We found that co-treatment with PP2 or dasatinib plus ATRA accelerated G1/0 arrest and upregulated expression of differentiation markers including CD11b and p47<sup>phox</sup>. Strikingly, we observed that ATRA increased c-Raf serine phosphorylation at S259 as well as residues in the C-terminal domain, and this effect was significantly enhanced with inhibitor co-

treatment. To our knowledge this is the first report of this phenomenon. These results suggest a role for c-RafpS259 in cell growth arrest, consistent with a study showing that a c-RafS259 mutation is proliferative and failed to induce PC12 differentiation compared to overexpression of activated wild-type c-Raf [46]. Similarly, increased c-Raf CTD phosphorylation is reported to enhance PC12 differentiation [47].

We also showed that combination treatment using either inhibitor plus ATRA increased the associations between Lyn, c-Raf, and c-RafpS259, as well as ERK and c-Raf. We detected interaction with the serine/threonine kinase CK2, which regulates various aspects of the cell cycle and is phosphorylated by SFKs, as well as binding with KSR1, a MAPK scaffold reported to modulate protein interactions to control signal intensity and duration [48-50]. The increase in c-Raf CTD phosphorylation suggested a KSR1-scaffolded signaling module that facilitates c-Raf/ERK interaction and creates a feedback loop where ERK may phosphorylate c-Raf to attenuate mitogenic signaling [47, 51].

We observed an interesting difference between the inhibitory effects of dasatinib and PP2 after ATRA treatment. Dasatinib was able to inhibit Lyn activity in the presence of ATRA while PP2 was not, yet both enhanced differentiation. The mechanism by which ATRA protects Lyn from the inhibitory effects of PP2 is unknown. The results indicate that Lyn kinase function is not required for differentiation. Therefore Lyn's role as an adaptor or scaffold seems to be more important, and points to its multi-functionality. In addition, it appears that CD38- and ATRA-driven tyrosine phosphorylation of c-Cbl is not necessary for differentiation. However, the c-Cbl TKB mutant crippled differentiation which indicates another function or modification of c-Cbl is involved that may be modulated by CD38. We did detect low-level, residual p85 PI3K phosphorylation after dasatinib treatment, which could be sufficient for its function, yet

significant downregulation did not seem to interfere with differentiation. Importantly, these results show that Lyn is the primary kinase involved in ATRA-induced pY-c-Cbl and pY-p85 PI3K. It is significant that PP2/ATRA treatment showed a pronounced enhancement in differentiation compared to dasatinib/ATRA co-treatment, and suggests that Lyn/CD38-driven tyrosine phosphorylation of c-Cbl and p85 PI3K may give PP2 the advantage over dasatinib in accelerating differentiation.

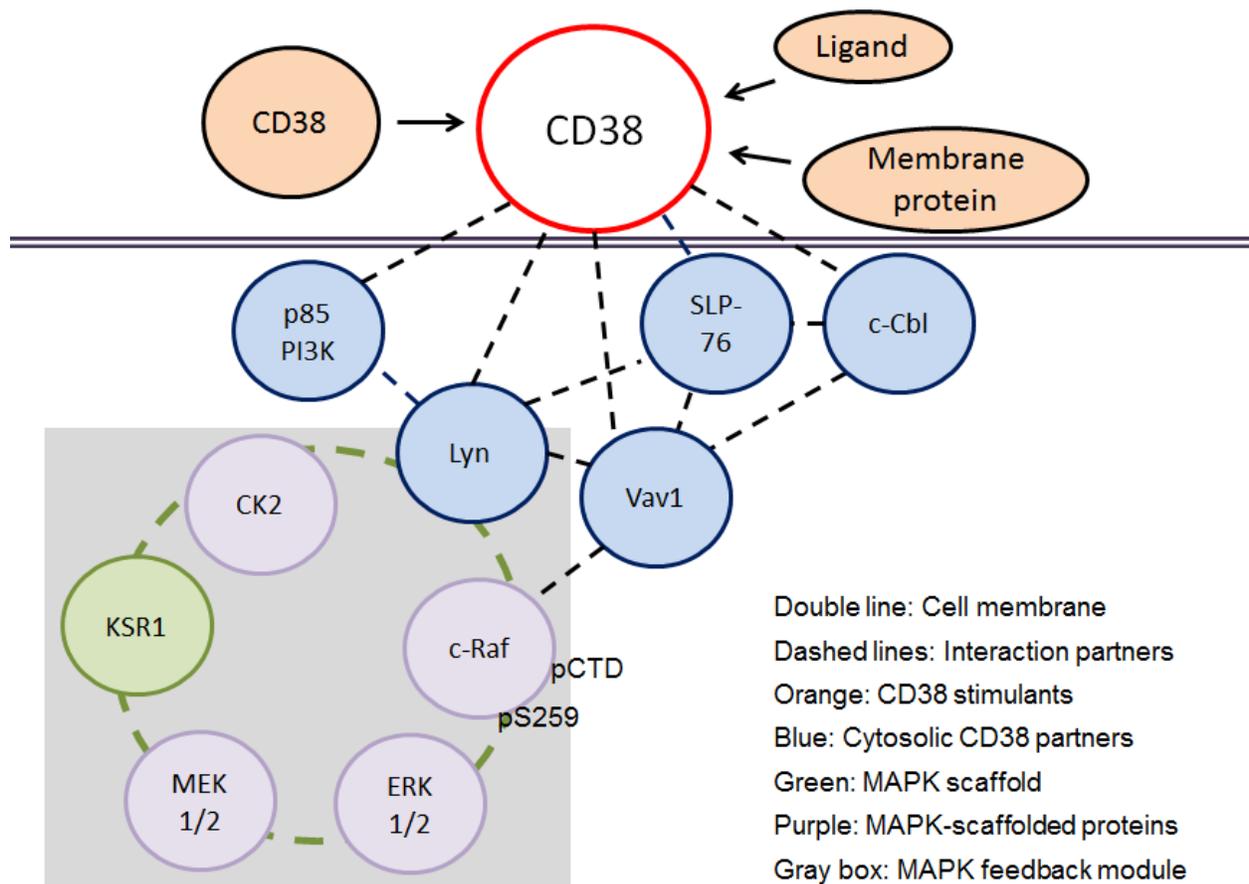
Future research could explore how ATRA protects Lyn activity and may reveal additional molecules that participate in augmenting the effects of ATRA and therapeutic targets. The multifunctionality of Lyn as a kinase and adaptor/scaffold is also of particular interest. PP2/ATRA co-treatment upregulates total Lyn protein expression which may enhance its ability to facilitate the previously described MAPK feedback signaling module. Since Lyn appears to be multifunctional CD38 may also be driving differentiation observed after ATRA- or PP2/ATRA co-treatment by participating in the proposed MAPK feedback complex. Since CD38 propels sustained MAPK signaling associated with differentiation, it may be an upstream regulator of the feedback module. Further research using CD38 knockdown or knockout models will provide deeper clarity regarding if and how CD38 is involved in the Lyn/c-Raf/ERK MAPK feedback complex. It is also of interest to further explore how CD38 and p-ERK cooperate to maintain a labile precommitment memory and if the associated, sustained MAPK signal involves the proposed KSR1-scaffold feedback complex.

In summary, the research presented here advances the understanding of how CD38, SFKs such as Lyn, and MAPK-associated molecules may drive ATRA induction. We show that CD38 receptor functions (versus enzymatic activity) are responsible for driving differentiation.

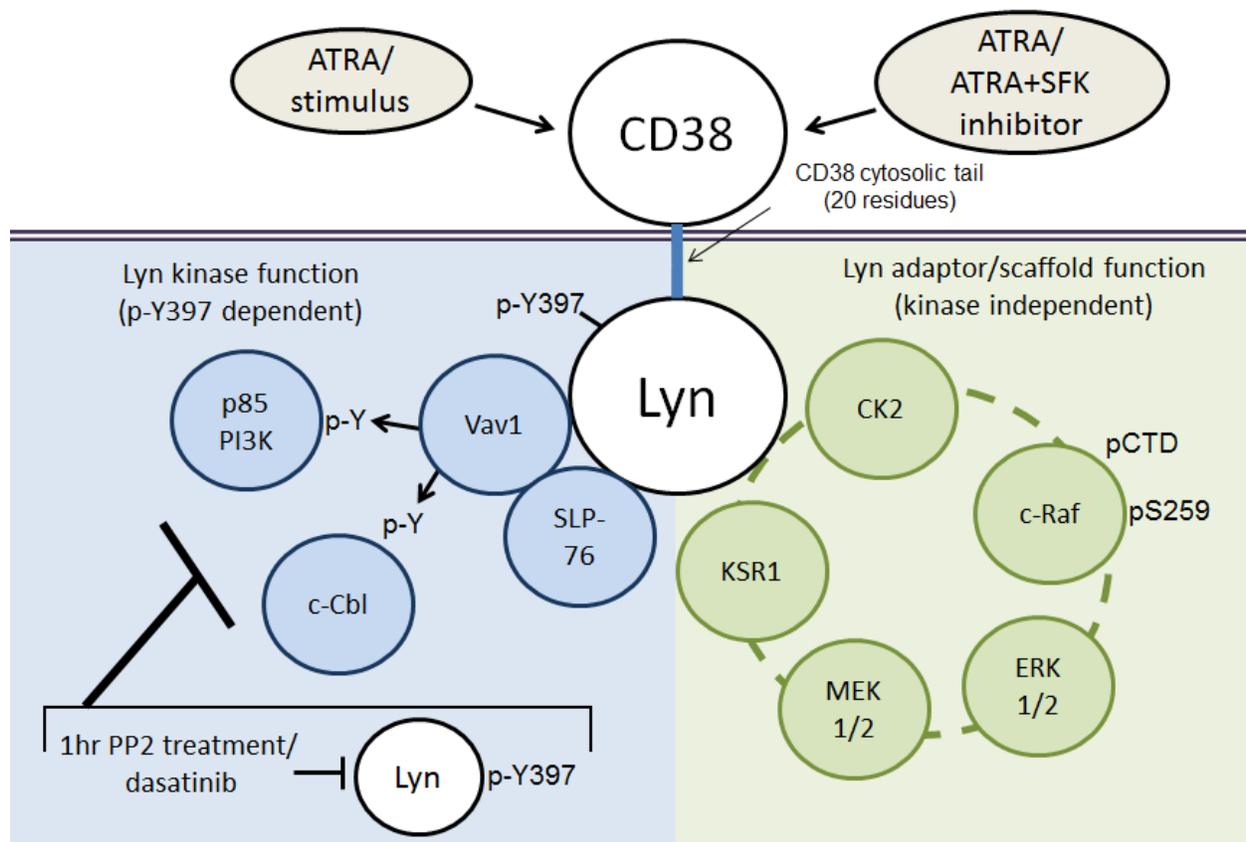
Receptor signaling appears to involve the assembly of a signaling complex containing SLP-76,

Vav1, and Lyn, which mediates ATRA- and CD38-stimulated signaling including phosphorylation of p85 PI3K and c-Cbl. These results support a body of data indicating that membrane-expressed CD38 interaction with the ATRA-regulated molecules c-Cbl, SLP-76, Vav1, p85 PI3K, and Lyn propels different signaling outcomes associated with induced myeloid differentiation. These interactions may also regulate the MAPK cascade, including the persistent signal that characterizes ATRA treatment and the newly reported MAPK feedback loop involving Lyn, c-Raf, and ERK (Figure 7.1). CD38 interaction with Lyn is of particular interest since Lyn appears to perform multiple functions associated with ATRA-induced differentiation. Lyn can serve as a kinase that is required for ATRA- and CD38-propelled c-Cbl and p85 PI3K phosphorylation in addition to adaptor/scaffold functions that may support the maintenance of the MAPK feedback loop reported here (Figure 7.2). Since CD38 and Lyn partner to drive signaling, it is possible that CD38 is also key regulator of the Lyn/c-Raf/ERK MAPK signaling module and may be a target of therapeutic interest.

We also show that SFK inhibitors can enhance the effects of ATRA, and that the positive MAPK feedback mechanism resulting post-translational modifications to c-Raf may be central to inhibitor augmentation of ATRA induction. This report has significant implications for the clinical use of SFK inhibitors which may compliment ATRA treatment. Ultimately these results suggest additional therapeutic options for resistant or relapsed patients and may improve the efficacy of ATRA in treating a variety of leukemias, and could expand its therapeutic use in other types of cancers.



**Figure 7.1 CD38/Lyn/MAPK interaction schematic.** ATRA-induced CD38 receptor signaling may be stimulated by ligands, membrane proteins, or dimer/oligomerization with itself. Downstream signaling events propelled by stimulation involve CD38 interaction partners that include p85 PI3K, Lyn, Vav1, SLP-76, and c-Cbl. CD38 may regulate MAPK signaling through its interaction with Lyn to drive a persistent cellular signal and the proposed Lyn/c-Raf/ERK feedback loop. Previous research shows that Vav1 also interacts with c-Raf showing that CD38 binds at least two MAPK signaling modulators that are also able to complex with c-Raf.



**Figure 7.2 Lyn participates in different signaling cascades that characterize ATRA-induced differentiation.** Lyn is a multifunctional protein that regulates signaling propelled by both ATRA and CD38. ATRA- and CD38-stimulated signaling (left) results in phosphorylation of c-Cbl and p85 PI3K and is dependent on Lyn kinase activity regulated by tyrosine phosphorylation of residue Y397. After 48 hours of ATRA treatment, inhibiting Lyn by one hour of PP2 incubation or 48 hours of dasatinib co-administration blocks c-Cbl and p85 PI3K phosphorylation and interrupts interaction between CD38/Lyn/Vav1/SLP-76. Lyn also functions as an adaptor/scaffold independent of kinase activity (right) and participates in a proposed MAPK feedback signaling module that may be regulated by CD38. 48 hours of co-treatment with ATRA plus SFK inhibitors that differentially regulate Lyn kinase activity enhances interaction between Lyn/c-Raf and c-Raf/ERK. CK2 and MEK 1/2 also participate in this complex which appears to be scaffolded by KSR1. Increased phosphorylation of c-Raf S259 and the CTD suggests a MAPK feedback module where ERK may phosphorylate c-Raf to attenuate mitogenic signaling and propel cell cycle arrest and differentiation.

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## **APPENDIX**

### **ADDITIONAL PUBLICATIONS WITH CONTRIBUTIONS BY THE AUTHOR**

**Mechanism-based small molecule probes for labeling CD38 on live cells.** Jiang H, Congleton J, Liu Q, Merchant P, Malavasi F, Lee HC, Hao Q, Yen A, Lin H. *J Am Chem Soc.* 2009 Feb 11;131(5):1658-9.

### **Abstract**

CD38 is a type II transmembrane glycoprotein with multiple functions. It acts as an ecto-enzyme as well as a receptor. The enzymatic activity catalyzes the formation of two potent  $\text{Ca}^{2+}$  releasing agents: cyclic adenosine diphosphate ribose (cADPR) from nicotinamide adenine dinucleotide (NAD) and nicotinic acid adenine dinucleotide phosphate (NAADP) from NAD phosphate (NADP). The receptor function of CD38 leads to the phosphorylation of intracellular signaling proteins and the up-regulation of cytokine production in immune cells. These two functions of CD38 underlie its involvement in various biological processes, such as hormone secretion, immune cell differentiation, and immune responses. Clinically, CD38 is used as a negative prognosis marker for chronic lymphatic leukemia (CLL). However, a clear molecular understanding of CD38's role in physiology and pathology is still lacking. To facilitate the study of CD38 at cellular and molecular levels, here we report a mechanism-based method for fluorescently labeling CD38 on live cells. This labeling method does not interfere with the receptor function of CD38 and the downstream signaling. The labeling method is thus a useful tool to study the receptor function of CD38 in live cells. In addition, since the mechanism-based labeling also inhibits the enzymatic activity of CD38, it should be useful for dissecting the receptor function of CD38 without interference from its enzyme function in complicated biological processes.

**Interferon regulatory factor-1 binds c-Cbl, enhances mitogen activated protein kinase signaling and promotes retinoic acid-induced differentiation of HL-60 human myelomonoblastic leukemia cells.** Shen M, Bunaciu RP, Congleton J, Jensen HA, Sayam LG, Varner JD, Yen A. *Leuk Lymphoma*. 2011 Aug 24.

## **Abstract**

Abstract All-trans retinoic acid (RA) and interferons (IFNs) have efficacy in treating certain leukemias and lymphomas, respectively, motivating interest in their mechanism of action to improve therapy. Both RA and IFNs induce interferon regulatory factor-1 (IRF-1). We find that in HL-60 myeloblastic leukemia cells which undergo mitogen activated protein kinase (MAPK)-dependent myeloid differentiation in response to RA, IRF-1 propels differentiation. RA induces MAPK-dependent expression of IRF-1. IRF-1 binds c-Cbl, a MAPK related adaptor. Ectopic IRF-1 expression causes CD38 expression and activation of the Raf/MEK/ERK axis, and enhances RA-induced differentiation by augmenting CD38, CD11b, respiratory burst and G0 arrest. Ectopic IRF-1 expression also decreases the activity of aldehyde dehydrogenase 1, a stem cell marker, and enhances RA-induced ALDH1 down-regulation. Interestingly, expression of aryl hydrocarbon receptor (AhR), which is RA-induced and known to down-regulate Oct4 and drive RA-induced differentiation, also enhances IRF-1 expression. The data are consistent with a model whereby IRF-1 acts downstream of RA and AhR to enhance Raf/MEK/ERK activation and propel differentiation.

## **Investigation of the c-Raf interactome and steady-state multiplicity in retinoic acid-induced differentiation of HL-60 cells.**

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### **Abstract**

Lessons learned in model differentiation systems, such as the lineage uncommitted human myeloblastic leukemia cell line HL-60, could inform the analysis of more complex differentiation programs. HL-60 undergoes myeloid differentiation and G1/0-arrest following persistent MAPK activation, when exposed to All-Trans Retinoic Acid (ATRA). The architecture responsible for ATRA-induced persistent MAPK activation, commitment, cell-cycle arrest and differentiation, as well as pretreatment memory effects, is poorly understood. In this study, we explored the ATRA-inducible cRaf interactome to determine the functional and regulatory architecture responsible for persistent MAPK activation in HL-60 cells. We surveyed a panel of 19 possible cRaf interaction partners in the presence or absence of ATRA and the cRaf inhibitor GW5074. We found five proteins (AKT, CK2, 14-3-3, Src and Vav1) that interacted with cRaf under different conditions. Of these five, the interaction between cRaf and Vav1 demonstrated a constant correlation with ATRA-inducible MAPK activation and aspects of functional differentiation. To better understand the role of cRaf in the ATRA-induced differentiation program, we constructed a family of mechanistic mathematical models of the transcriptional and post-translational events driving persistent ATRA-induced MAPK activation. The proposed architecture was consistent with experimental observation. Bifurcation analysis of this model family predicted bistability in ppERK levels as a function of ATRA forcing. A

functional consequence of this non-linear behavior was the ability to lock the MAPK cascade into a self-sustaining activated state, even in the absence of ATRA. These simulations were qualitatively validated with ATRA washout experiments. The results provide further details on sustained MAPK activation, mechanistic insight for aspects of cellular memory, and proof-of-concept that a combination of experimental and computational methods is an effective strategy for dissecting complex intracellular programs.