**Evolution of Acute Myelogenous Leukemia Stem Cell Properties Following
Treatment and Progression**

by

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Submitted in Partial Fulfillment of the

Requirements for the Degree

Doctor of Philosophy

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Rochester, New York

2014

Biographical Sketch

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The following publications were a result of work conducted during doctoral study:

Tzu-Chieh Ho, Mark W. LaMere, Kristen O'Dwyer, Jason H. Mendler, Jane L. Liesveld, Meir Wetzler, Eunice S. Wang, Monica L. Guzman, Craig T. Jordan and Michael W. Becker. Evolution of acute myelogenous leukemia stem cell properties following treatment and progression. In submission.

Tzu-Chieh Ho, Michael W. Becker. Defining patient-specific risk in acute myeloid leukemia. J Clin Oncol. 2013 Nov 1;31(31):3857-9.

Acknowledgements

First I would like to acknowledge my advisors Dr. Michael W. Becker and Dr. Craig T. Jordan. I am very lucky having them as my role models. They show me not only how to do research, but also how to be a good scientist. Furthermore, they help me walk through this long journey, and I really enjoy the time working in the lab. They are absolutely my best mentors. Certainly, I have to thank the members of my committee: Dr. Jane Liesveld, Dr. James Palis, and Dr. Margot Mayer-Pröschel. They are always nicely guiding and teaching me, and I am very thankful for their help and support. In addition, I would like to thank Dr. Mark Noble. In the two years under NYSTEM training grant, Dr. Noble taught me a lot how to be a good researcher. I also want to thank all the collaborators in this project, especially Dr. John Ashton and Dr. Jason Mendler. They are always willing to share their valuable thoughts with me. Moreover, I am so grateful to all the past and present members in Becker lab, Jordan lab and cancer center for their advice and friendship, especially Mark, Lizz, Minhaj, Sarah, Marlene, and Maya. Last but not least, I deeply thank my family and Dennis Jiang for their support and love. After these precious years, we finally see the light in the end of tunnel, and I do hope to become the person that makes you proud.

Abstract

Acute myeloid leukemia (AML) is a malignant hematopoietic disease characterized by abnormal proliferation and differentiation of neoplastic hematopoietic cells. The leukemia stem cell model proposes that a small population of leukemic cells, termed leukemic stem cells, LSCs, are responsible for maintaining the disease and serve as a reservoir for relapse following treatment. To date no study has formally assessed the evolution of LSC population(s) following therapy. To address this critical question, we performed a prospective characterization of paired primary human AML samples at diagnosis and in relapse in a well-defined cohort of patients to assess the frequency, surface antigen phenotype and molecular signatures of functionally defined LSCs. We demonstrate by limiting dilution analyses (LDA) a 9 to 90-fold increase in LSC frequency between diagnosis and relapse in paired primary AML samples. The increase in LSC activity was not associated with an increase in the frequency of phenotypically defined populations previously reported to possess LSC activity. Using xenotransplantation assay, LSCs were identified to expand in relapse to phenotypically distinct populations (as defined by CD34 and CD38 or CD32 and CD38) that did not possess LSC activity prior to treatment. Further, while the majority of the LSC populations' gene expression profile remained stable between diagnosis and relapse, a subset of genes was dysregulated in functionally defined LSC populations at relapse including interleukin-1 receptor accessory protein (IL1RAP), previously demonstrated to play a role in LSC biology. Overexpression of IL1RAP was demonstrated on the cell surface of bulk and functionally defined LSC populations in the majority of AML

specimens at diagnosis and relapse. IL1RAP knockdown increased cell death as well as reduced cell proliferation, clonogenicity and engraftment potential of primary AML and U937 cells, implicating an important role of IL1RAP in AML. Preliminary drug treatment and RNA-sequencing assays suggest the possibility of IL1RAP to regulate LSC/progenitor population through NF- κ B signaling. Taken together, our data suggest that conventional chemotherapy for AML patients may promote quantitative and qualitative expansion of the LSC compartment. Understanding the impact on LSC signatures following therapy may help to develop new AML regimens in future studies.

Contributors and Funding Sources

This work was supervised by dissertation committee consisting of Dr. Michael W. Becker of the Department of Medicine, Hematology/Oncology at the University of Rochester Medical Center and Dr. Craig T. Jordan of the Department of Hematology at the University of Colorado School of Medicine, and Dr. Jane Liesveld of the Department of Medicine, Hematology/Oncology, Dr. James Palis of the Department of Pediatrics, Hematology and Oncology and Dr. Margot Mayer-Pröschel of the Department of Biomedical Genetics at the University of Rochester Medical Center. The cell sorting depicted in the dissertation was performed by either URM C Flow Cytometry Core facility (FCC) with the assistance of Sarah J Neering. The TLDA analyses for Chapter 2 and RNA-seq analyses for Chapter 3 were conducted by University of Rochester Genomics Research Center (URGRC). The immunophenotyping for Chapter 2 and mouse works depicted in the dissertation were conducted in collaboration with Mark W. LaMere. All other work conducted for the dissertation was completed by the student independently. Graduate study was supported by the University of Rochester Stem Cell and Regenerative Medicine Institute (SCRMI) NYSTEM Training Grant from the Empire State Institutional Training Program in Stem Cell Research. The research was funded by the following award: University of Rochester Clinical Translational Institute Award and R21CA149848-01 PI: Michael Becker, M.D., PI.

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List of Abbreviations

ABC.....	ATP-Binding Cassette
ADCC.....	Antibody-Dependent Cell-mediated Cytotoxicity
ALL.....	Acute Lymphoblastic Leukemia
AML.....	Acute Myelogenous Leukemia
Ara-C.....	Cytarabine
BCR-ABL.....	Breakpoint Cluster Region/Abelson Kinase translocation product
BM.....	Bone Marrow
CD34.....	Cluster of Differentiation antigen 34
CD38.....	Cluster of Differentiation antigen 38
CDC.....	Complement-Dependent Cytotoxicity
cDNA.....	Complementary DNA
CFU.....	Colony Forming Unit
ChIP-seq.....	Chromatin Immunoprecipitation Sequencing
C-MAP.....	Connectivity Map
CML.....	Chronic Myelogenous Leukemia
CR.....	Complete Remission
DAPI.....	4', 6 diamidino-2-phenylindole
DEG.....	Differentially Expressed Gene
DFS.....	Disease Free Survival
DMEM.....	Dulbecco's Modified Eagle Medium
DNA.....	Deoxyribonucleic Acid

DNR.....	Daunorubicin
FAB.....	French–American–British classification
FACS.....	Fluorescence-Activated Cell Sorting
FBS.....	Fetal Bovine Serum
FISH.....	Fluorescent <i>in situ</i> Hybridization
FLT3.....	Fms-Like Tyrosine kinase 3
gDNA.....	Genomic DNA
GCSF.....	Granulocyte Colony-Stimulating Factor
GFP.....	Green Fluorescent Protein
GMP.....	Granulocyte-Macrophage Progenitors
HiDAC.....	High-Dose Cytarabine
HSC.....	Hematopoietic Stem Cell
IL-1.....	Interleukin 1
IL-1R.....	Interleukin 1 receptor
IL-1ra.....	IL-1 receptor antagonist
IL1RAP.....	Interleukin-1 receptor accessory protein
IL-3.....	Interleukin 3
IL-33.....	Interleukin 33
IL-7.....	Interleukin 7
IMDM.....	Iscoe’s Modified Dulbecco’s Medium
IP.....	Intraperitoneal
IRB.....	Institutional Review Board

ITD.....	Internal Tandem Duplication
IVIG.....	Intravenous Immune Globulin
LAIP.....	Leukemia-Associated Immunophenotype
LDA.....	Limiting Dilution Analysis
LICs.....	Leukemia-Initiating Cells
Lin ⁻	Lineage Negative
LSC.....	Leukemia Stem Cell
LSC-R.....	LSC-Related gene signature
mAb.....	Monoclonal Antibody
MeV.....	Multiexperiment Viewer
MDS.....	Myelodysplastic Syndrome
MFI.....	Median Fluorescence Intensity
MLL.....	Myeloid/Lymphoid or Mixed-Lineage Leukemia
MNC.....	Mononuclear Cells
MRD.....	Minimal Residual Disease
NBM.....	Normal Bone Marrow
NF- κ B.....	Nuclear Factor- κ B
NK.....	Natural Killer
NOD/SCID.....	Non-Obese Diabetic/Severe Combined Immunodeficiency
NSG.....	NOD/SCID/interleukin 2 receptor γ chain null
NSGS.....	NSG-SGM3
OS.....	Overall Survival

PB.....	Peripheral Blood
PBS.....	Phosphate Buffered Saline
PEG.....	Polyethylene Glycol
PES.....	Polyethersulfone
PI3K.....	Phosphatidylinositol 3' Kinase
PTD.....	Partial Tandem Duplication
qRT-PCR.....	Quantitative Real-Time Polymerase Chain Reaction
RFS.....	Relapse-Free Survival
RNA.....	Ribonucleic Acid
RNAi.....	RNA Interference
RNA-seq.....	RNA Sequencing
SCF.....	Stem Cell Factor
SFM.....	Serum Free Medium
shRNA.....	Short Hairpin Ribonucleic Acid
SIRP α	Signal Regulatory Protein alpha
TIM-3.....	T cell Immunoglobulin Mucin-3
TLDA.....	TaqMan [®] Low Density Array
UHRR.....	Universal Human Reference RNA
WGS.....	Whole Genome Sequencing

Chapter 1

Introduction

1.1 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is a malignant hematopoietic disease characterized by abnormal proliferation and differentiation of neoplastic hematopoietic cells, and results in impaired hematopoietic function such as neutropenia or anemia (Shipley & Butera, 2009; Tallman, Gilliland, & Rowe, 2005). Several factors are linked to the prognosis and clinical outcome, including patient age, genetic and epigenetic alterations (e.g., chromosomal changes, overexpression or mutations) (Shipley & Butera, 2009; Tallman et al., 2005). Patient age is an important prognostic factor for AML as increasing age worsens the prognosis with standard therapy (Shipley & Butera, 2009). Genetic and epigenetic abnormalities are related to the development of AML. Approximately 60% of AML patients have an abnormal karyotype at diagnosis (Grimwade & Hills, 2009). Based on the cytogenetics, AML patients are classified into three risk groups (favorable, intermediate and adverse) for prognosis (Grimwade & Hills, 2009). Not all patients with a normal karyotype have the same clinical outcome (Shipley & Butera, 2009). This may be due to various genes overexpressed and/or mutated, which frequently happen in AML. Multiple genetic alterations with prognostic significance have been characterized, including internal tandem duplication of the *FLT3* gene (FLT3-ITD), partial tandem duplication of the *MLL* gene (MLL-PTD), mutations in the *NPM1* gene and *CEBPA* gene, and high expression of the *WT1*, *EVII*, and *BAALC* genes (Grimwade & Hills, 2009; Mrozek, Marcucci, Paschka, Whitman, & Bloomfield, 2007; van Rhenen, Moshaver, Ossenkoppele, & Schuurhuis, 2007).

1.2 Standard chemotherapy for AML

Currently the standard approach to treating patients with AML includes induction chemotherapy designed to restore hematopoiesis followed by consolidation chemotherapy. The induction chemotherapy typically consists of a combination of the cell cycle-specific agent cytarabine 100 mg/m^2 by continuous intravenous infusion for 7 days and the anthracycline antibiotic daunorubicin 45 to 90 mg/m^2 for 3 days (“7+3” regimen) or idarubicin 12 mg/m^2 (Shipley & Butera, 2009; Tallman et al., 2005). Following the induction chemotherapy is consolidation chemotherapy with high-dose cytarabine $2\text{-}3 \text{ g/m}^2$ (HiDAC), which is applied with the hope of eliminating minimal residual disease (Shipley & Butera, 2009; Tallman et al., 2005). Approximately 80% of adult AML patients achieve complete remission (CR) after induction chemotherapy. However, without consolidation chemotherapy, >90% of AML patients will suffer relapse. Even with consolidation chemotherapy, over 50% of patients eventually relapse and die after successful induction and consolidation therapies. Only 30% to 40% of patients have long-term disease free survival (DFS) (Shipley & Butera, 2009; Tallman et al., 2005).

1.3 The leukemia stem cell model in AML

An accurate definition of a cancer stem cell is based on its functional properties, which means a malignant cell has the abilities to undergo self-renewal and differentiation to recapitulate the original cell populations found in the given tissue (Jordan, 2009). The leukemia stem cell model postulates that a rare LSC population exists within AML and is

responsible for maintaining the disease. A decade ago, Dick group clearly identified LSCs in human AML by xenotransplantation with immunodeficient SCID or NOD/SCID mice, and demonstrated that LSCs were capable of differentiating to leukemic blasts *in vivo*, indicating AML is hierarchically initiated from LSCs (Bonnet & Dick, 1997; Lapidot et al., 1994). The origin of LSCs is less clear. One model suggests that mutations targeting the normal hematopoietic stem cell (HSC) and transform a pool of HSCs into its malignant counterpart, LSC (Rosen & Jordan, 2009). Thus, it is plausible that HSCs and LSCs share properties, such as stem cell immunophenotype and ability to undergo aberrant differentiation and maturation. However, HSCs may not be the only origin of LSCs. Another model suggests that some cancer genes are able to transform myeloid non-self-renewing progenitor cells into LSCs in AML (Krivtsov et al., 2006; Passegue, 2006). Certain oncogenic events (e.g., MLL-AF9) target committed progenitor cells and confer self-renewal ability upon these normally non-self-renewing populations (Krivtsov et al., 2006). A second issue is that features of LSC are not stably exhibited in all patients as it is in the normal system (Jordan, 2009). The LSC properties such as immunophenotype, cell frequency and response to extrinsic stimuli vary among patients, and may change during disease progression or after treatment (Jordan, 2009). Thus, all experimentation which aims to examine leukemia stem cells must include studies of functionality in addition to immunophenotypes.

1.4 Drug resistance in LSCs

One proposed consequence of the leukemia stem cell model is that standard chemotherapies do not effectively eradicate LSCs and these residual cells are responsible for disease relapse. If so, therapies that can target and ablate LSCs will increase the chance to eliminate the cancer in patients. Demonstration of a greater degree of resistance to standard chemotherapy agents by AML LSCs in contrast to non-LSC populations has been reported. LSCs are less sensitive to cytarabine (Ara-C) or anthracycline (e.g., daunorubicin) in comparison with more mature leukemic blasts (Costello et al., 2000; Guzman et al., 2001). In addition, these chemotherapy agents have shown no selectivity for LSCs versus HSCs (Costello et al., 2000; Guzman et al., 2005; Jordan, 2007). A potential mechanism for this resistance is the relative quiescence of LSCs, like HSCs, resulting in less response to cell cycle-specific cytotoxic chemotherapy (e.g., Ara-C) (Guan, Gerhard, & Hogge, 2003; Guan & Hogge, 2000; Ishikawa et al., 2007). Another mechanism by which LSCs are less sensitive to standard therapies is the increased expression of genes/proteins related to membrane drug efflux transporters or apoptosis resistance to response drug stimuli (Dean, Fojo, & Bates, 2005). For example, 22 ATP-binding cassette (ABC) transporter genes such as MDR1 (ABCB1) and BCRP (ABCG2) were highly differentially expressed in AML LSCs ($CD34^+CD38^-$) compared with more committed leukemic cells ($CD34^+CD38^+$) (de Grouw et al., 2006). Similar results were also shown in normal hematopoietic cells (de Grouw et al., 2006). Such ABC transporters can rapidly pump chemotherapy drugs out of cells and are thought to be involved in multidrug resistance in AML. Since the LSCs properties are easily influenced by genetic

aberrancies, disease progression and drug stimuli (Rosen & Jordan, 2009), it is possible that more mechanisms are able to confer drug resistance in LSCs, which is worth further study.

1.5 The immunophenotype in AML LSC

LSCs have been reported as a rare population in AML blasts (Eppert et al., 2011; Sarry et al., 2011), which is a limitation for studying LSC specific signatures or properties during patients' clinical course. An approach to isolate LSCs from abundant AML bulk cells is using flow cytometry/sorter, which is able to identify surface markers specifically expressed on the LSCs. In the original papers by Dick group, AML LSCs were contained within the CD34⁺CD38⁻ fraction of cells for the majority of patients (Bonnet & Dick, 1997; Lapidot et al., 1994). The choice to study CD34 and CD38 was based on the experience of studying normal HSCs. These initial studies supported a model in which the LSCs arose from transformation of the normal HSC population. However, recent studies using novel immunodeficient models of xenotransplantation have challenged this hypothesis and determined that LSC phenotype is more heterogeneous than previously thought. A report by Taussig et al. demonstrated the limitations of NOD/SCID xenograft assays. Treatment of leukemic cells with anti-CD38 antibodies prior xenotransplantation inhibited leukemic engraftment through the antibody-dependent cell-mediated cytotoxicity (ADCC) effects from the residual natural killer (NK) cells in NOD/SCID mice (Taussig et al., 2008). Using an improved xenograft system, LSCs were

demonstrated to exist within CD34⁺CD38⁺ or CD34⁺ fractions in different types of AML (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2010).

In recent years, a number of antigens expressed on AML cells have been reported (Jordan, 2010; Majeti, 2010). Many of them have been validated by xenotransplantation assays and were shown to be expressed on functional LSCs. Such markers include CD123 (Jin et al., 2009; Jordan et al., 2000), CD44 (Jin, Hope, Zhai, Smadja-Joffe, & Dick, 2006), CLL-1 (van Rhenen, van Dongen, et al., 2007), CD96 (Hosen et al., 2007), CD47 (Majeti, Chao, et al., 2009), CD25 (Saito et al., 2010), CD32 (Saito et al., 2010), CD45RA (Goardon et al., 2011) and TIM-3 (Kikushige et al., 2010). CD123 (IL3R alpha chain) is the first marker identified to be strongly expressed on LSCs (98±2% in 16 out of 18 primary AML CD34⁺CD38⁺ samples), whereas no significant expression is found on highly enriched human HSC populations (<1% in 5 out of 5 normal CD34⁺CD38⁺ samples) (Jordan et al., 2000). Functional xenograft assays showed that the CD123⁺ populations were capable of engrafting in NOD/SCID mice in all cases (n = 3) (Jordan et al., 2000). Moreover, the CD123⁺ populations did not contribute to engraftment in all but one case (n = 3) (Jordan et al., 2000). However, the utility of this marker on LSC discrimination is reduced after conventional chemotherapy as CD123 is up-regulated on regenerating bone marrow CD34⁺CD38⁺ cells following chemotherapy (median = 60%, n = 6) (van Rhenen, Moshaver, Kelder, et al., 2007). CD44 is hyaluronan receptor that can mediate cell adhesion (Avigdor et al., 2004) and intracellular signal transduction (Turley, Noble, & Bourguignon, 2002). The expression of CD44 on the CD34⁺CD38⁺ fraction of AML was enhanced (n = 8) compared to normal human cord blood cells (n = 4-5) (Jin et

al., 2006). An activating monoclonal antibody (mAb) specific to CD44 (H90) has been shown to block homing and induce differentiation of AML LSCs leading to a significant reduction of leukemic repopulation in NOD/SCID xenograft assays (Jin et al., 2006).

CD96, Tactile, is a member of the Ig gene superfamily that is expressed on activated T cells (P. L. Wang, O'Farrell, Clayberger, & Krensky, 1992). CD96 is also highly expressed on AML CD34⁺CD38⁻ cells (74.0±25.3% in 19 out of 29 samples) but only about 5% of normal HSCs (CD34⁺CD38⁻CD90⁺Lin⁻) (in 3 out of 3 samples) (Hosen et al., 2007; Majeti, 2010). CD47 is an immunoglobulin-like protein that is capable of interacting with its receptor SIRPα (signal regulatory protein alpha). SIRPα is expressed on macrophages and dendritic cells and its binding inhibits phagocytosis (Brown & Frazier, 2001; Jaiswal et al., 2009; Majeti, Chao, et al., 2009). CD47 was more highly expressed on AML LSCs (CD34⁺CD38⁻CD90⁻Lin⁻) than on normal bone marrow HSCs (CD34⁺CD38⁻CD90⁺Lin⁻), and is an adverse prognostic factor of AML (Majeti, Chao, et al., 2009). A monoclonal antibody against CD47 enables phagocytosis of AML LSCs and inhibits the engraftment *in vivo* (Majeti, Chao, et al., 2009). CD25 (IL2R alpha chain) and CD32 (Fc-gamma receptor II) expression on normal cells are both restricted to the hematopoietic system (Majeti, 2010; Saito et al., 2010). In patients with AML, CD25 and CD32 were found to be differentially expressed on LSCs (CD34⁺CD38⁻) (mean fluorescence intensity (MFI) > 800 in 15 and 21 out of 61 samples, respectively) compared to normal HSCs (MFI±SD is 162.8±32.6 and 103.6±34.4 in 5 normal CD34⁺CD38⁻ samples, respectively) (Saito et al., 2010). These markers were used to identify cell cycle-quiescent AML LSCs lining the BM endosteal surface following Ara-

C treatment in a xenotransplantation model for human AML (Saito et al., 2010). A recent study found that AML patients with CD25 expression of >10% has significantly shorter overall survival (OS) and relapse-free survival (RFS), suggesting the role of CD25 as an independent adverse prognostic marker in AML patients ≤ 60 years (Terwijn et al., 2009). CD45RA is an isoform of CD45 that functions as a negative regulator of some cytokine signaling (Seita & Weissman, 2010). In human, HSCs are within Lin⁻CD34⁺CD38⁻CD90⁺CD45RA⁻ population (Majeti, Park, & Weissman, 2007). A recent paper showed the CD34⁺ cells in ~80% of human AML specimens they tested contained two predominant populations, CD38⁻CD90⁻CD45RA⁺ and CD38⁺CD110⁺CD45RA⁺, which both have LSC activity (Goardon et al., 2011). TIM-3 (T cell immunoglobulin mucin-3) is expressed on monocytes and a part of natural killer (NK) cells in the periphery, but not normal HSCs or the majority of CD34⁺CD38⁺ progenitor cells except a small fraction of granulocyte-macrophage progenitors (GMPs) with low TIM-3 expression level (Kikushige et al., 2010). However, TIM-3 is expressed on LSCs (CD34⁺CD38⁻) as well as leukemic progenitor cells (CD34⁺CD38⁺) in most AML FAB types except M3 (Kikushige et al., 2010). ATIK2a, a mAb against TIM-3 with potent complement-dependent (CDC) and antibody-dependent cellular cytotoxic (ADCC) activities has been shown to block engraftment of AML after xenograft studies (Kikushige et al., 2010).

In addition to the above surface antigens, Schuurhuis group characterized AML blast aberrant phenotypes at diagnosis and following relapse to develop a flow cytometric platform for monitoring minimal residual disease (MRD). They then employed a panel of

antigens to identify patient specific leukemia-associated immunophenotypes (LAIPs) (Schoorhuis & Ossenkoppele, 2010; van Rhenen, Moshaver, Kelder, et al., 2007; van Rhenen, Moshaver, Ossenkoppele, et al., 2007). The LAIPs included lineage markers (CD7, CD11b, CD13, CD19, CD33, and CD56, etc.), CD123 and CLL-1 (van Rhenen, Moshaver, Kelder, et al., 2007). MRD cells are leukemic cells that persist following chemotherapy and the outgrowth of MRD cells was thought to be responsible for relapse (Feller et al., 2004; Kern et al., 2004; San Miguel et al., 2001; van Rhenen et al., 2005; Venditti et al., 2000). Using the LAIPs, the MRD frequency after chemotherapy can be determined by flow cytometry, and the level of MRD assessed in this manner correlates with the incidence of relapse and survival making the level of MRD following therapy an independent prognostic factor for relapse-free and overall survival in AML (Feller et al., 2004; Kern et al., 2004; San Miguel et al., 2001; van Rhenen, Moshaver, Kelder, et al., 2007; Venditti et al., 2000).

1.6 LSC specific signatures

Given the failure of current therapy to cure most individuals with AML, there is a significant need for different approaches. Towards this aim, LSC specific signatures have been employed to identify potential novel targets for developing new therapies. An ideal regimen should exclusively eradicate LSCs but not impair normal HSCs. Thus, it is important to understand how LSCs differ from HSCs. A variety of studies have identified LSC specific signatures and some therapeutic agents have been developed based on these studies. These involve mediating cell survival or response to injury (e.g., regulate

oxidative state) (Gilliland, Jordan, & Felix, 2004; Jordan, Guzman, & Noble, 2006). For example, two cell survival mechanisms, nuclear factor- κ B (NF- κ B) and phosphatidylinositol 3' kinase (PI3K) pathways, both have been reported to be constitutively active in AML LSCs but neither pathway has significant activity in normal HSCs (Guzman et al., 2001; Xu, Simpson, Scialla, Bagg, & Carroll, 2003). Inhibition of NF- κ B rapidly induced AML LSC death with minimal impact on normal HSCs (Guzman et al., 2001; Guzman et al., 2005). Similarly, inhibition of PI3K leads to a decrease in survival of AML LSCs (Xu et al., 2003). Our lab has previously characterized the leukemic progenitor/LSC specific signature using Affymetrix arrays and bioinformatics technology (Majeti, Becker, et al., 2009). This genome-wide expression analysis directly compared the expression profile of highly enriched AML CD34⁺CD38⁻CD123⁺Lin⁻ cells with normal HSCs (CD34⁺CD38⁻CD90⁺Lin⁻). Multiple LSC specific pathways were identified; some have been previously or subsequently published related to drug resistance, including Wnt signaling pathways and apoptosis pathway, etc. In addition to LSC specific signatures dysregulated between LSCs and HSCs, Eppert et al. generated a LSC specific signature (LSC-R) by comparing global gene expression of LSC-enriched populations to non-LSC populations and identified a core transcriptional program shared by LSCs and HSCs (Eppert et al., 2011). They further demonstrated the LSC-R signature was correlated with poor clinical outcome in AML with normal karyotype (Eppert et al., 2011). Taken together, targeting LSC specific signatures seems a feasible strategy for developing a new regimen to combat AML.

Chapter 2

Evolution of Acute Myelogenous Leukemia Stem Cell Properties Following Treatment and Progression

2.1 Abstract

Background

Acute Myelogenous Leukemia (AML) evolves over time as patients who are responsive to therapy upfront frequently acquire resistance to the same agents when applied in the relapse setting. The stem cell model for AML has been invoked to explain primary resistance to standard therapy; the leukemia stem cell (LSC) population representing a therapy-refractory reservoir for relapse; however, there have been no prospective efforts to formally assess the evolution of the LSC population during patients' clinical course. We performed a prospective characterization of specimens from a well-defined cohort of patients with AML at diagnosis and relapse to assess the frequency and phenotype of functionally defined LSCs.

Methods

Primary bone marrow and peripheral blood samples were collected on Institutional Review Board (IRB) approved protocols from patients with newly diagnosed AML undergoing induction therapy. Twenty-five patients who relapsed after achieving a morphologic remission were selected for further study. Screening studies identified seven patients whose pre-therapy samples demonstrated sustained engraftment in NOD/SCID/interleukin 2 receptor γ chain null (NOD/SCID/IL2 $\gamma^{-/-}$, termed NSG) mice following xenotransplantation. Pre-therapy and post-relapse LSC frequencies were assessed using NSG xenotransplantation limiting dilution analyses (LDA). Paired pre-therapy and post-relapse LSC populations were identified using fluorescent-labeled cell

sorting based on expression of CD34 versus CD38 or CD32 versus CD38 and NSG xenotransplantation. LSC activity was confirmed for each population using secondary xenotransplantation. We then examined the frequencies of CD34, CD38 and CD123, CD33, CD97, CD32, CD47, CD96, TIM-3, CD45RA, and HLA-DR expressing populations on functionally validated LSC populations at diagnosis and relapse. Gene expression analysis of highly enriched LSC populations from pre-therapy and post-relapse samples was performed using Custom TaqMan[®] Low Density Array (TLDA) qPCR analyses following pre-amplification.

Results

We demonstrated by LDA a 9 to 90-fold increase in LSC frequency between diagnosis and relapse. The increase in LSC activity was not simply associated with an increase in frequency for phenotypically defined populations previously reported to possess LSC activity. Rather, we found that LSC activity expanded at relapse to immunophenotypic populations of leukemic cells (as defined by CD34 and CD38 or CD32 and CD38) that did not possess LSC activity prior to treatment. Further, while the majority of the LSC populations' gene expression profile remained stable between diagnosis and relapse, a subset of genes were enriched in defined LSC populations at relapse including IL1RAP, previously demonstrated to play a role in LSC biology (Askmyr et al., 2013; Barreyro et al., 2012; Jaras et al., 2010).

Conclusions

This study is the first to characterize the evolution of LSCs *in vivo* following treatment and relapse. We demonstrate an increase in LSC activity and greatly increased phenotypic diversity of the LSC population, suggesting a loss of hierarchical organization following relapse. These findings suggest that treatment of AML patients with conventional chemotherapy regimens may promote quantitative and qualitative expansion of the LSC compartment. Further, the data indicate that surface antigen immunophenotype is not predictive of function in relapse, and suggest a major limitation to efforts targeting specific surface antigens in the relapse setting. Understanding the mechanisms by which LSC expansion occurs and how to target it will likely improve our currently poor treatment options for patients who relapse.

2.2 Introduction

In acute myelogenous leukemia (AML), approximately 70% of adult patients achieve complete remission after standard chemotherapy with cytarabine and an anthracycline, “7+3”. To maintain remission, additional therapy either in the form of chemotherapy or stem cell transplant is required. Despite numerous attempts to advance treatment over the last three decades, most patients will experience disease relapse (Tallman et al., 2005). In contrast to upfront therapy, the response rates for relapsed AML are considerably lower and only a small fraction of patients is cured (Forman & Rowe, 2013). It has long been recognized that in patients with AML, leukemic “blasts” are not a uniform population of cells. Studies have shown that leukemic blasts differ in their response to chemotherapeutic challenge as well as in their ability to maintain the disease upon transplantation. The leukemia stem cell (LSC) model proposes that a rare population of leukemia cells maintains the disease and represents a therapy-refractory reservoir for relapse. Understanding the mechanisms by which LSCs can evade therapeutic challenge has the potential to improve our treatments for this disease. We and others have reported the transcription signatures for LSCs in comparison to non-LSC or normal HSCs (Eppert et al., 2011; Guzman et al., 2001; Majeti, Becker, et al., 2009). We identified 3,005 differentially expressed genes (DEG) and dysregulated signaling in highly enriched leukemic stem cell populations by genome-wide expression analysis (Majeti, Becker, et al., 2009). Eppert et al. generated a separate signature shared between AML LSC and normal HSC populations. This signature was highly correlated with overall survival in AML patients (Eppert et al., 2011). Although these findings suggest the potential

importance of LSC specific signatures, little is known of their evolution following treatment and disease progression.

LSCs have historically been identified and isolated based on their surface antigen expression profiles. CD34 and CD38 are the most frequently employed cell markers (Seita & Weissman, 2010). Initial publications supported the restriction of LSC capacity to the CD34⁺CD38⁻ population (Bonnet & Dick, 1997; Lapidot et al., 1994), however more recent reports have challenged this hypothesis demonstrating that human AML LSC phenotypes are more heterogeneous (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2008; Taussig et al., 2010). In addition to CD34 and CD38, additional surface markers that allow for LSC segregation include CD123 (Jin et al., 2009; Jordan et al., 2000), CD32 (Saito et al., 2010), CD33 (Taussig et al., 2005), CD45RA (Goardon et al., 2011), CD47 (Majeti, Chao, et al., 2009), CD96 (Hosen et al., 2007) and TIM-3 (Kikushige et al., 2010). However, the question of whether LSC phenotype is stable during patients' clinical course and contributes to relapse has not been clarified.

We hypothesized that if the LSC compartment represents a reservoir for relapse, the population would be present and stable throughout the clinical course. To address this hypothesis, we investigated the frequency, surface antigen phenotype and molecular signatures of functionally defined LSCs in paired primary human AML samples at diagnosis and in relapse. Surprisingly, we found that LSC frequency and phenotypic diversity are much greater at relapse, unlike LSC-specific gene expression profiles which remained largely stable. Our data suggest that current AML therapeutic regimens promote dramatic changes in the LSC compartment. A better understanding of how these

changes occur will aid in the development of novel therapeutics for patients with relapsed disease.

2.3 Materials and Methods

Patients

Twenty-five AML patients who relapsed after achieving a morphologic remission following standard induction therapy were selected for this study. Detailed patient clinical and pathologic data are provided in Table 2.1. Bone marrow aspirates or peripheral blood samples were obtained following informed consent at diagnosis and in relapse under protocols approved by the Institutional Review Board (IRB) at the relevant institutions (University of Rochester Medical Center (URMC) and Roswell Park Cancer Institute). Normal bone marrow (NBM) aspirates were obtained following informed consent on an URMC IRB approved protocol from healthy donors. All samples were processed using a standardized procedure. Cytogenetic analyses (G-banding and fluorescent *in situ* hybridization (FISH)) were performed as clinically indicated. Mononuclear cells (MNCs) were harvested using a Ficoll gradient (GE Healthcare) and viably frozen in CryoStor[®] CS10 (BioLife Solutions) followed by storage in liquid nitrogen. Samples were thawed at 37°C in RPMI-1640 with L-glutamine (Lonza) plus 20% heat-inactivated fetal bovine serum (HI-FBS) (HyClone) and 1% Penicillin-Streptomycin (Gibco). After thawing, the average cell viability was >50%.

Mutation analysis

Genomic DNA (gDNA) was purified from bulk AML cells at diagnosis and relapse using AllPrep DNA/RNA Mini Kit (Qiagen). For samples with sufficient material, the mutational status of 33 genes (ASXL1, ATM, BRAF, CBL, CDKN2A, DDX3X,

DNMT3A, ETV6, EZH2, FBXW7, FLT3, GNAS, IDH1, IDH2, JAK2, KIT, KLHL6, KRAS, MAPK1, MYD88, NOTCH1, NPM1, NRAS, PTEN, PTPN11, PHF6, RUNX1, SF3B1, TET2, TP53, WT1, XPO1, ZMYM3) were determined by multiplex PCR and next generation sequencing using the Hematologic Malignancy Sequencing panel developed by the Center for Personalized Diagnostics at the University of Pennsylvania on an Ion Torrent Personal Genome Machine (PGM) platform.

Immunophenotyping

Thawed primary AML cells were stained with mouse anti-human antibodies against CD34 (PE-Cy7, 8G12, BD BioSciences), CD38 (APC, HB7, BD BioSciences or PerCp-Cy5.5, HIT2, BD Pharmingen), and leukemia specific aberrant markers. Leukemia specific aberrant markers include CD33 (FITC, HIM3-4, BD Pharmingen), CD45 (PE-Cy5, HI30, BioLegend), CD123 (PE, 7G3, BD Pharmingen) and HLA-DR (APC-Cy7, L243, BD BioSciences) or CD32 (APC, 6C4, eBioScience), CD45RA (APC-Cy7, HI100, BioLegend), CD47 (APC, B6H12, eBioScience), CD96 (PE, NK92.39, eBioScience), CD97 (FITC, VIM3b, BD Pharmingen), and TIM-3 (PE, F38-2E2, BioLegend). 4', 6 diamidino-2-phenylindole (DAPI) (Invitrogen) were used to exclude non-viable cells. Flow cytometry was performed on a LSR II (BD Biosciences). For each specimen 3×10^5 to 1×10^6 events were analyzed. Data analyses were conducted using FlowJo software for Mac OS X (TreeStar, Ashland, OR) to determine the frequency of defined populations relative to live cell gate. Normal bone marrow samples were analyzed using the same approach.

Fluorescence-activated cell sorting

Paired primary AML samples at diagnosis and relapse were stained using anti-CD3 (PE-Cy5, HIT3a, BioLegend), anti-CD34 and anti-CD38 antibodies with DAPI in PBS plus 0.5% HI-FBS. Anti-CD3, anti-CD32 and anti-CD38 antibodies were employed for sorting when CD34 expression was absent. Viable T-cell-depleted AML cells (Dapi⁻CD3⁻ gating) were sorted under sterile conditions for CD34⁺CD38⁺, CD34⁺CD38⁻, CD34⁻CD38⁺ and CD34⁻CD38⁻ or CD32⁺CD38⁺, CD32⁺CD38⁻, CD32⁻CD38⁺ and CD32⁻CD38⁻ populations using a FACS Aria cell sorter (BD Biosystems). The purity of each sort was subsequently assessed and verified (84% to 100%). Sorted cells were collected in PBS plus 0.5% HI-FBS prior to transplant. For TaqMan[®] Low Density Array based analysis, cells were double-sorted into TRIzol[®] Reagent (Life Technologies, Invitrogen). Normal HSCs were isolated from thawed human normal BM samples enriched for CD34-positive cells using MACS Indirect CD34 MicroBead Kit and MACS Columns (Miltenyi Biotec). Enriched CD34-positive cells were then stained with biotinylated lineage antibodies against CD3 (UCHT1, BD Pharmingen), CD14 (61D3, eBioScience), CD15 (HI98, eBioScience), CD16 (3G8, BD Pharmingen), CD19 (HIB19, BD Pharmingen), CD56 (B159, BD Pharmingen) and Streptavidin (PE-Cy5, BD Pharmingen), as well as antibodies against CD34 (FITC, 581, BD Pharmingen), CD38 (PerCP-Cy5.5, HIT2, BD Pharmingen), CD90 (APC, 5E10, BD Pharmingen), CD123 (PE, 7G3, BD Pharmingen), glycophorin A (PE-Cy5, GA-R2, BD Pharmingen) and DAPI. Normal HSC cells (CD34⁺CD38⁻Lin⁻CD90^{low}) were further double-sorted into TRIzol[®] Reagent (Life

Technologies, Invitrogen) by using a FACS Aria cell sorter (BD Biosystems).

Limiting dilution analysis (LDA)

For xenograft studies, eight to ten week old NOD/SCID/interleukin 2 receptor γ chain null (NOD/SCID/IL2 $\gamma^{-/-}$, termed NSG) recipient mice received a dose of radiation (250 cGy) using a RadSource X-Ray irradiator 24 hours prior to transplantation. Two doses of human intravenous immune globulin (IVIG) were administered 0.5 mg/g by intraperitoneal (IP) injection on the day of irradiation as well as the day of transplantation. (Total dose is 1 mg/g). Primary bulk AML cells were thawed and serially diluted to obtain cell doses ranging from 1×10^3 to 5×10^6 . Cells were then transplanted into NSG mice via tail-vein route. For samples with fewer than one million cells per animal, syngeneic donor (NSG) splenocytes were mixed with the primary AML sample prior to transplantation. Engraftment was measured at 12 weeks post transplantation or at anytime that they appeared moribund as described above. The frequency of LSCs was calculated using software with the number of positively engrafted mice at each dose (L- Calc™ Software, STEMCELL Technologies).

Functional assessment of LSC activity on sorted populations.

Sorted cells representing four populations (CD34⁺CD38⁻, CD34⁺CD38⁺, CD34⁻CD38⁺, CD34⁻CD38⁻ or CD32⁺CD38⁻, CD32⁺CD38⁺, CD32⁻CD38⁺, CD32⁻CD38⁻) as well as unsorted cells were injected into mice via tail vein injection (final volume of 0.2 mL). The number of cells transplanted for each sorted population was proportional to the

contribution of that population to the patient's original sample. The equivalent dose of mononuclear cells (MNCs) per mouse was 5×10^5 to 3×10^6 cells for sorted populations. Unsorted group was injected with 1×10^6 cells per mouse. Sorted populations with fewer than one million cells per animal were mixed with splenocytes prior to transplantation as above. Mice were euthanized at 12 weeks post transplantation or anytime they appeared moribund. Mouse bone marrow (BM) was analyzed by flow cytometry and human engraftment was defined by the percentage of human CD45⁺ staining cells greater than 0.1% of live cells in sample. Recipient BM cells were also stained with antibodies specific to mouse CD45 (APC, 30-F11, BD Pharmingen), human CD19 (PE, HIB19, BD Pharmingen) and human CD97 (FITC, VIM3b, BD Pharmingen) to remove murine cells and ascertain the tumor burden. CD97 was selected in place of CD33 given a greater degree of surface antigen density. Mice demonstrating over 50% of human CD45⁺ events positive for CD19 and lacking CD97 expression were classified as having normal engraftment. The expression of other antigens (CD123, CD33, TIM-3, CD47, CD96, CD32, CD45RA and HLA-DR) was also performed to confirm the leukemic engraftment.

Serial transplantation assay

To document the capacity for sustained self-renewal, secondary transplantation studies were performed for all populations demonstrating leukemic engraftment when possible. One to five million unsorted bone marrow cells from xenotransplanted mice where human leukemic engraftment was documented as above were transplanted into irradiated NSG mice as above. Mice were sacrificed 12 weeks post secondary transplantation or

anytime that they appeared moribund. Recipient BM cells were analyzed as above to assess the leukemic engraftment.

Custom TaqMan[®] Low Density Array (TLDA) based gene expression analysis

Total RNA of normal donor human HSC populations and functionally defined LSC populations were isolated from TRIzol[®] Reagent (Life Technologies, Invitrogen) and cleaned-up with RNeasy column (Qiagen). cDNA was synthesized using iScript[™] cDNA Synthesis Kit (Bio-Rad) and pre-amplified with pooled TaqMan[®] Gene Expression Assay primer/probe combinations and TaqMan[®] PreAmp Master Mix (Life Technologies, Applied Biosystems). Custom TaqMan[®] Low Density Array (TLDA) cards were designed to contain 156 genes selected from our previous microarray data (Majeti, Becker, et al., 2009) and Eppert et al. (Eppert et al., 2011). Levels of gene expression from independent runs were normalized using an universal human reference RNA (UHRR) (Stratagene) standard. UHRR is composed of total RNA from 10 different human cell lines and designed to be used as a constant reference in microarray gene-profiling analyses. The qRT-PCR was carried out using the Applied Biosystems 7900HT Sequence Detection System (Life Technologies, Applied Biosystems). The analysis was performed using ExpressionSuite Software (Life Technologies, Applied Biosystems) with *GAPDH* and *HPRT1* to normalize expression.

Quantitative Real-Time PCR (qRT-PCR)

For individual gene analyses, qRT-PCR was performed using TaqMan[®] Gene Expression

Assay, TaqMan[®] PreAmp Master Mix and TaqMan[®] Gene Expression Master Mix (Life Technologies, Applied Biosystems) and carried out on the LightCycler[®] 480 Real-Time PCR System (Roche).

Bioinformatic analysis

Immunophenotyping data were analyzed for their relevance using two-tailed Mann–Whitney test (NBM vs. AML) or Wilcoxon matched-pairs signed rank test (diagnosis vs. relapse) for continuous variables. *P* value < 0.05 was considered as significant. TLDA and qRT-PCR data were analyzed for their relevance using two-tailed Wilcoxon matched-pairs signed rank test and Student's t-test for continuous variables. *P* value < 0.05 was considered as significant. Clustering and heat maps were generated using Multiexperiment Viewer (MeV). LSC and HSC data were clustered using Pearson correlation metric with average linkage. For comparisons between the gene expressions of relapse and diagnosis samples, a fold change cutoff of 1.5 was used in analyses.

2.4 Results

The LSC frequency is increased between diagnosis and relapse

The stem cell model for leukemia proposes that LSCs are a rare population in patients with AML. To confirm the rarity of this population as well as to directly assess the impact of therapy on LSC frequency, we performed limiting dilution analyses (LDA) on unsorted bulk cells from 5 paired primary patient samples at diagnosis and in relapse (Table 2.1 and 2.2). Similar to previously published results, we demonstrate that LSCs are rare with considerable inter-patient heterogeneity in frequency (range 1 in 9×10^3 to 1 in 2.3×10^6 cells) in the pre-therapy samples. At relapse, LSCs still represent a small fraction of the leukemic mass with frequencies varying between one in $<1 \times 10^3$ to one in 1×10^5 cells. Direct comparison of pre-therapy and relapse samples for individual patients demonstrated a previously unreported 9 to 90-fold increase in LSC frequency (Table 2.2). These results show for the first time a significant increase in the LSC pool following relapse in patients with AML.

Little change in the expression of leukemia stem cell markers from diagnosis to relapse

CD34 and CD38 are frequently employed to identify LSC populations from primary human AML samples. We examined the expression of CD34 and CD38 on primary BM or PB specimens from 25 AML patients at diagnosis and relapse. Representative examples of leukemic and normal BM are shown in Figures 2.1A and 2.1B. While the expression of CD34 and CD38 varied little between normal donors (Figure 2.1A), AML

cases demonstrated a wide range of expression profiles (Figure 2.1B and 2.1C) in agreement with prior studies (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2010). Within a patient, the profile was generally stable from diagnosis to relapse (Figure 2.1B and 2.1C) despite the marked increase in LSC activity identified by LDA.

Increased diversity of LSC phenotype at relapse

To functionally assess the impact of treatment and relapse on the phenotype of the patient's specific LSC populations, all 25 pre-treatment samples were assessed for ability to engraft in NSG mice. We identified 7 of the 25 samples capable of engrafting NSG mice with sufficient material to perform detailed analyses of LSC activity. Four individual populations, based on the expression of CD34 and CD38 (Figure 2.2A) or CD32 and CD38 expression were sorted and transplanted into sublethally irradiated NSG recipients (Figure 2.2B and 2.2C). CD32 was employed in cases where CD34 expression was absent (Figure 2.2D). CD32 is a transmembrane glycoproteins expressed on B cells, granulocytes, monocytes, macrophages, and platelets that plays a role in the interaction between Fc gammaRII-expressing cells and antigens. CD32 was previously reported to mark the LSC populations of some patients with AML (Saito et al., 2010) and CD32 and C38 routinely segregate leukemic cells into four populations. Purity checks on all post-sorting cells averaged at least 95%. We observed LSC activity not only within CD34⁺CD38⁻ population, but also within other populations, confirming early reports that the LSC phenotype is heterogeneous between patients (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2010). Interestingly, the majority of patients demonstrated LSC

activity in more than one population at diagnosis. Neither CD34 and CD38 nor CD32 and CD38 expression was universally associated for LSC phenotype at diagnosis.

Examining LSC activity at relapse, we surprisingly identified a previously unreported expansion of LSC activity to immunophenotypic populations of leukemic cells that did not possess LSC activity prior to treatment (Figure 2.2B and 2.2C) regardless of the antigens tested (CD34, CD32 or CD38). In six out of seven patients, the number of phenotypically distinct LSC populations (as defined by CD34 and CD38 or CD32 and CD38) was expanded post-relapse. For example, LSC ability was detected in CD34⁺CD38⁺ and CD34⁺CD38⁻ populations in AML3 at diagnosis, but expand to all four populations following treatment and disease progression. Furthermore, the leukemic burden of recipient mice was higher in relapse samples compared to diagnosis samples for the paired samples that we injected similar/more amount of equivalent MNCs to recipient mice at diagnosis compared to relapse (AML1, AML3, AML22, and AML18) (Figure 2.2C). Overall, these data demonstrate that LSCs become more phenotypically diverse following treatment and progression. The increased level of NSG engraftment at relapse suggests that the increase in LSC frequency identified in our LDA is distributed across all cell populations.

Distinct LSC populations contain long-term self-renewal ability and recapitulate AML phenotype in xenografts

To confirm the self-renewal capability of distinct LSC populations, we performed secondary transplantation studies for all populations demonstrating leukemic engraftment

(Figure 2.3A and Table 2.3). All samples with a sufficient level of engraftment in the primary recipient were capable of leukemic engraftment of secondary recipients. We also verified the ability of transplanted sorted populations to recapitulate the phenotype of the unsorted sample in xenografts (Figures 2.3B and 2.4). For AML3, engrafted cells derived from primary $CD34^+CD38^+$ population reconstituted human $CD34^+CD38^+$ phenotype in first xenograft BM. It also recapitulated other human sub-populations in mice (such as $CD34^+CD38^-$ population). Similarly, xenografts transplanted with primary $CD34^+CD38^-$ population gave rise to not only human $CD34^+CD38^-$ cells but other CD34 and CD38 populations (such as $CD34^+CD38^+$ population) as well. In samples absent CD34 expression (AML18 and AML25), the expression of CD34 was still absent in the engrafted cells derived from each sorted population, while CD38 was highly expressed as in the primary AML specimens (Figures 2.4).

Surface antigen immune-phenotype is not predictive of function in relapse

Multiple reports have identified antigens associated with functional LSC activity including CD32, CD33, CD45RA, CD47, CD96, CD123 and TIM-3 (Goardon et al., 2011; Hosen et al., 2007; Jordan, 2010; Jordan et al., 2000; Kikushige et al., 2010; Majeti, 2010; Majeti, Chao, et al., 2009; Saito et al., 2010; Taussig et al., 2005). Several of these antigens are being developed for LSC targeted therapies including CD33, CD123 and CD47. To determine if the increase in LSC frequency we identified between diagnosis and relapse was associated with the expression of a previously reported LSC antigen, we assessed the expression of the above antigens on primary BM or PB

specimens from 25 AML patients at diagnosis and relapse (Figure 2.5). CD123, CD33, CD97, TIM-3 and HLA-DR expression readily distinguished bulk leukemic cells from normal BM cells (Figure 2.5A) while CD123, CD97, TIM-3 and CD45RA readily distinguished leukemic CD34⁺CD38⁻ cells and CD34⁺CD38⁺ cells from their normal counterparts (Figure 2.5B and 2.5C). Only the frequency of CD123-positive cells in the CD34⁺CD38⁻ population was significantly increased between diagnosis and relapse (70.2±28.1% vs. 87.4±15.1%, p=0.0048). We then examined the expression of the same LSC antigens on functionally defined LSC as well as non-LSC fractions from 7 AML patients at diagnosis and in relapse (Figure 2.6A). When assessing CD32 expression, we analyzed populations only from patients with LSC activity defined by the expression of CD34 and CD38 (Figure 2.6A). CD123, CD33, CD97 and TIM-3 readily allowed LSCs to be distinguished from normal HSCs. Analysis of the twelve non-LSC populations from diagnosis which developed LSC activity at relapse (Figure 2.6B) demonstrated that only TIM-3 and CD45RA expressing cells increase in frequency during the evolution from diagnosis to relapse (26.29±24.66% vs. 50.86±33.37%, p = 0.0342 and 31.97±25.75% vs. 59.02±34.41%, p = 0.0322, respectively). The approximately two fold-change increase in expression of TIM-3 and CD45RA on LSCs between diagnosis and relapse is well below the 9 to 90-fold increase in LSC activity identified by LDA. These findings suggest that the increase in LSC frequency between diagnosis and relapse is independent of the expression of previously reported LSC antigens.

Identification of molecular mechanisms associated with drug treatment and

progression in AML LSC

Previous studies have attempted to gain insight into LSC signaling through gene expression profiling of enriched LSC populations (Eppert et al., 2011; Majeti, Becker, et al., 2009; Saito et al., 2010). In our previous study, we compare signaling profiles of leukemic $CD34^+CD38^-CD123^+Lineage^-$ cells to normal HSC signaling with the goal of identifying differences that would represent potential therapeutic targets (Majeti, Becker, et al., 2009). We identified 3,005 DEGs and 10 pathways dysregulated in enriched LSC populations ($CD34^+CD38^-CD123^+Lin^-$) compared to normal HSC populations ($CD34^+CD38^-CD90^+Lin^-$) using genome-wide expression analyses and two independent pathways analyses (Majeti, Becker, et al., 2009). More recently, Eppert et al. performed an analysis in which they compared the expression profiles of functionally defined LSC populations to non-LSC populations regardless of surface antigen phenotype (Eppert et al., 2011). To characterize the impact of treatment and relapse on LSC signatures, we analyzed the expression profile of functionally defined LSC fractions at diagnosis and relapse using targeted expression analysis. 123 of the 3005 DEGs were selected for study based on their role in five of the dysregulated pathways (Adherens Junction, Wnt, Regulation of Actin Cytoskeleton, Jak-STAT, and MAPK) or a reported role in response to therapy. 27 genes were selected from a 42 gene LSC-related gene (LSC-R) signature reported by Eppert et al. (Eppert et al., 2011). 10 of these 27 genes were also present our 3,005 DEG list (Figure 2.7A). Gene expression values were normalized to a universal human reference RNA (UHRR) (Stratagene). Results of molecular signatures in 12 pairs of functionally defined LSC populations (from 7 different AML patients) are shown in

Figure 2.7. We observed significant inter-patient heterogeneity between AML populations most notable for patients with LSCs defined by CD32 and CD38 (Figure 2.7B). Hierarchical clustering demonstrated that LSC populations from individual patients were more likely to cluster together than diagnosis or relapse samples from different patients (Figures 2.7B). Comparing the gene expression pattern of diagnosis and relapse LSCs within individual patients, the majority of LSC signature was maintained from treatment to progression. However, a subset of genes, 5 out of 15, was up-regulated in relapse across the majority of specimens (fold change ≥ 1.5 ; ≥ 6 out of 12 populations and 4 out of 7 patients) regardless of their phenotype, including *ATP1B1*, *SERPINE1*, *C16orf5*, *FLJ13197*, *MAFF* and *IL1RAP* (Figure 2.7C and 2.7D). The genes were divided between our previously reported signature and the signature reported by Eppert et al. We also identified a second subset of genes down-regulated in relapse (fold change ≤ 0.67 ; ≥ 6 out of 12 populations and 4 out of 7 patients), including *BCL6*, *WASF3*, *ITGB2*, *IL6ST*, and *IRAK3* (Figure 2.7C and 2.7D). This gene set consisted entirely of genes selected from the 3005 DEGs except *BCL6*. These results confirm our prior findings of dysregulated signaling in LSC populations from patients with AML. Interestingly, despite the marked increase in LSC frequency, there was little variation in the gene signature analyzed in this study whether based on our original signature or the signature of Eppert et al. (Eppert et al., 2011; Majeti, Becker, et al., 2009).

2.5 Discussion

The leukemia stem cell model is routinely summoned to explain why the majority of patients relapse following initial therapy for AML. We hypothesized that if the LSC compartment represents a therapy insensitive reservoir for disease relapse, the population would be present and identifiable throughout the clinical course. We therefore investigated the frequency, surface antigen phenotype and molecular signatures of functionally defined LSCs in paired primary human AML samples at diagnosis and in relapse. While LSC frequency has been reported to vary considerably between individual patients (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2010), prior reports have not examined the impact of therapy and relapse on LSC frequency or phenotype. By employing limiting dilution analyses, the gold standard for assessing stem cell frequency, we were able to identify a previously unreported 9 to 90-fold increase in LSC frequency following relapse. By assessing the LSC activity in populations defined by either CD34 and CD38 or CD32 and CD38, we demonstrate an expansion of the LSC activity to populations of cells resembling a more differentiated/mature phenotype at relapse irrespective of the antigens used to define the populations. We also found an increase in the level of leukemic engraftment of NSG recipients, a surrogate for LSC frequency, for individual populations from the majority of patients at relapse. LSC expansion was not associated with an increased frequency of CD34, CD38 expression or most of the previously published LSC associated antigens. While the frequency of TIM-3 and CD45RA expression rose two –fold, this increase was well below the 9 to 90-fold increase in LSC activity observed on LDA. Thus, the frequency of previously reported

LSC antigens, do not appear to track with LSC activity in the course of patients' disease.

Since the original report in 1997 by Bonnet et al. (Bonnet & Dick, 1997), the surface antigen expression profiles of functional AML LSC populations have become increasingly heterogeneous. In normal hematopoiesis, CD34 and CD38 expression defines the degree of myeloid differentiation and maturation. Assuming the relationships of CD34, CD32 and CD38 remain intact in AML, these findings suggest that in different patients, LSC phenotypes range from that of an HSC to that of late progenitors. The basis of this inter-patient heterogeneity remains largely undefined. Studies in mice demonstrate that the phenotype of functional LSC populations is in part dependent upon the stage of myeloid development targeted by the leukemia initiating events. Non-self-renewing normal hematopoietic progenitors are transformed into LSCs given the right set of genetic events (Krivtsov et al., 2006). In human AML, we are just beginning to dissect the stage of myeloid development targeted. Populations of cells termed pre-leukemic stem cells resemble HSCs and appear to be the reservoir for initial events. Additional events target later progenitors giving rise to the clinical disease. Data from whole genome sequencing may help to address the clonal structure of the disease during the disease course.

When we examined LSC signaling following relapse, HSC populations clustered independently of LSC populations consistent with dysregulated signaling in the malignant stem cell populations. Sorted populations from the same patient were more likely to segregate with each other as opposed to disease state (diagnosis vs. relapse) highlighting inter-patient heterogeneity. When assessing individual genes, most

demonstrated little change between diagnosis and relapse. The genes up-regulated following relapse in at least one LSC population in a minimum of 4 out of 7 patients include *ATP1B1*, *SERPINE1*, *C16orf5*, *FLJ13197*, *MAFF* and *IL1RAP*. While little has been reported a functional role for either *ATP1B1* or *SERPINE1* in LSC function, *SERPINE2* (PAI-2), has been shown to be important for leukemia growth and LSC development (Ashton et al., 2012). *FLJ13197* has been reported to be overexpressed in hematopoietic stem cells (Georgantas et al., 2004; Toren et al., 2005); no prior studies have examined its function in malignant stem cell populations. *IL1RAP* overexpression has been reported recently in chronic myelogenous leukemia (CML) (Jaras et al., 2010), AML (-7/7q-, normal karyotype) and high-risk myelodysplastic syndrome (MDS) patients (Barreyro et al., 2012). *IL1RAP* overexpression is associated with poor overall survival in AML patients with normal karyotype (Barreyro et al., 2012). Antibodies against *IL1RAP* have been shown to kill CD34⁺CD38⁻ populations in CML (Jaras et al., 2010) and AML (Askmyr et al., 2013) samples. Interestingly, *IRAK3* is down-regulated in functionally defined LSC populations between diagnosis and relapse. A member of the interleukin-1 receptor-associated kinase protein family, *IRAK3* is a negative regulator of Toll/IL-R immune signaling. We demonstrated that *IL1RAP* is overexpressed on functionally defined LSC populations compared to normal HSCs (Figure 2.7D). Functional validation studies are underway to define the role of *IL1RAP* and IL-1 signaling in LSC function.

In chronic myelogenous leukemia, patients in chronic phase may experience a transformation of their disease to an acute leukemia resembling AML called blast crisis.

In chronic phase, the “leukemic stem cell” population has a surface antigen phenotype minimally distinguishable from that of a normal HSC. In 2004, Jamieson et al. demonstrated leukemic stem cell activity in cells with a surface antigen phenotype resembling that of the granulocyte-macrophage progenitors (GMPs) in patients with blast crisis CML (Jamieson et al., 2004). The Jamieson lab went on to show that a potential mechanism for this expansion was related to activation of WNT signaling in more committed progenitors. Activation of the WNT pathway is known to be involved in self-renewal decisions for both normal and malignant stem cell populations. Using qPCR TLDA, we examined signaling for five of the pathways we previously identified as being dysregulated in LSC populations, including the WNT pathway. We did not identify enrichment of WNT signaling in the pre-therapy and post-relapse LSC populations using this approach. Eppert et al. recently reported on the LSC-R “self-renewal “signature from functionally validated LSC populations from AML patients (Eppert et al., 2011). Only three of the 27 genes selected from their 42 gene signature differed from diagnosis to relapse despite the marked expansion of LSC activity.

Of the genes up-regulated at relapse, IL1RAP alone has been shown to have a role in LSC function. IL1RAP is a co-receptor for IL-1 signaling and cell autonomous IL-1 signaling has been demonstrated to result in decreased apoptosis in AML blasts (Turzanski, Grundy, Russell, & Pallis, 2004). IL-1 signals through two distinct pathways, the NF- κ B pathway as well as the MAPK/c-Jun pathway. While we did not demonstrate enhanced MAP kinase signaling in LSCs following relapse by gene expression profiling, we did not examine NF- κ B signaling. Recently, activation of the NF- κ B pathway via

TNF- α signaling has been reported to be critical for LSC expansion in multiple murine models for AML as well as in human AML samples (Kagoya et al., 2014). In addition, NF- κ B is increasingly being recognized as a critical co-factor for MLL (Kuo et al., 2013). Whether IL1RAP signaling through NF- κ B is directly involved in the expansion of LSC activity following relapse is currently under investigation.

Our findings have consequences in several areas including the validity of the leukemic stem cell model. Our analyses of pre-therapy samples are consistent with the prior efforts of Bonnet, Dick, and Carroll in that LSC activity can be identified in populations of leukemic blasts with a surface antigen phenotype characteristic of not only early progenitors but committed progenitors (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2008; Taussig et al., 2010). On relapse, although LSC frequency remains rare, CD34 and CD38 or CD32 and CD38, were no longer useful for segregation of LSC activity from cells lacking this capacity, a requirement for cancer stem cells.

In the last decade, a considerable effort has been made to identify cell surface LSC specific targets against which to direct therapies. These efforts have included CD33, CD123 as well as CD47 (Bross et al., 2001; Frankel, Liu, Rizzieri, & Hogge, 2008; Majeti, Chao, et al., 2009). We demonstrated that the CD33 and CD123 failed to consistently correlate with the increase in LSC activity at relapse while CD47 was incapable of distinguishing functional LSCs from normal stem cells. The failure of the studied LSC markers to consistently track with the LSC expansion identified on LDA predicts that they will not effectively target all LSC populations, especially in the relapse setting where most novel therapeutics are employed.

Table 2.1. Characteristics of AML diagnosis and relapse specimens

AML	Blast (%)	WBC (K)	Cytogenetics	Mutation (Diagnostic sample)
AML1	60-65% BM, 21% PB	80	Normal	FLT3 INS
AML2	44% BM, 4% PB	18.4	Normal	NPM1 INS, ASXL1 SNP, WT1 INS
AML3	74% BM, 64% PB	13.1	42-44XY, t(3;16), del5q?31,-9,-17,-18, +2mar	FLT3 INS, TP53 DEL
AML4	Inspirable 11% PB	17.9	Normal	DNMT3A SNP, FLT3 SNP, NPM1 INS, TET2 DEL
AML5	Inspirable 55% PB	40.2	46,XX,t(11;19)(q23;p13.1)[20]	ASXL1 SNP, FLT3 SNP, KRAS SNP, MLL INS, PTEN SNP, TET2 SNP, TET2 SNP, TP53 SNP
AML6	70% BM	31.9	Normal	ASXL1 SNP, FLT3 ITD, DNMT3A SNP, KIT SNP, NPM1 INS, TET2 SNP, TET2 SNPSNP, TET2 SNP
AML7	60% BM	10.9	Normal	(NPM1)
AML8	21% BM	4.1	Normal	RUNX1 SNP, TET2 SNP
AML9	83% BM	5.2	46,XX,+1,der(1;15)(q10;q10)[18]/46,XX[2]	ND
AML10	81% BM	171.6	Normal	FLT3 INS, NPM1 INS, DNMT3A SNP
AML11	91% BM	54.5	+8, inv(9) (p21q21)?c	Flt3 INS
AML12	56% BM	92.2	inv (16)(p13q22)	NRAS SNP
AML13	90% BM	118.1	del(20)(q11q13)	EZH2 SNP, TET2 INS, RUNX1 SNP, FLT3 SNP
AML14	48% BM	11.4	Normal	ND
AML15	39% BM	3.7	Normal	FLT3 INS, NPM1 INS, DNMT3A SNP, NRAS SNP
AML16	54% BM	77.4	t(9;22)(q34;q11)	ETV6 SNP
AML17	49% BM	14.5	Normal	TET2 SNP, ASXL1 SNP
AML18	94% BM	68.5	Normal	NPM1 INS, TET2 INS/SNP, DNMT3A SNP
AML19	77% BM	77.7	+11	RUNX1 INS, FLT3 SNP, ATM SNP
AML20	28% BM	3.2	Normal	ASXL1 INS/SNP, IDH2 SNP, CBL SNP
AML21	93% BM	178.3	del(16)(q22q24)	ASXL1 SNP, FLT3 INS, ATM SNP
AML22	80% BM	29.5	+8	TET2 DEL, IDH1 SNP, NRAS SNP
AML23	46% BM	91.3	ins(8;8)(q22;q13q24)	TET2 SNP, RUNX1 SNP, IDH2 SNP
AML24	29% BM	49.7	+2mar	NPM1 INS, TET2 SNP, DNMT3A SNP
AML25	87% BM	95.8	45,X,-Y,t(9;11)(p22;q23)	Flt3INS, Flt3 SNP

Table 2.2. Results of limiting dilution analysis from bulk cells of 5 pairs of AML patient samples at diagnosis and relapse

AML	Status	No. of cells injected								Lower limit*	Frequency	Upper limit*
		1E3	5E3	1E4	5E4	1E5	5E5	1E6	5E6			
AML1	Diagnosis	----	----	----	0/4	0/3	3/4	3/3	1/1	1,005,514	428,455	182,567
AML1	Relapse	----	----	3/3	5/5	5/5	5/5	5/5	4/4		<10,000	
AML3	Diagnosis	----	3/5	2/4	5/5	5/5	5/5	3/3	----	20,977	8,815	3,704
AML3	Relapse	4/4	5/5	5/5	5/5	5/5	5/5	2/2	----		<1,000	
AML15	Diagnosis	----	----	0/5	0/5	1/5	1/5	1/2	4/5	5,500,772	2,297,888	959,918
AML15	Relapse	0/5	1/5	1/5	1/4	3/5	5/5	2/2	----	205,081	92,862	42,049
AML18	Diagnosis	----	1/5	1/4	3/5	2/3	4/4	1/1	----	126,550	56,116	24,884
AML18	Relapse	4/5	5/5	3/3	4/4	4/4	----	----	----	1,809	619	212
AML22	Diagnosis	----	0/2	1/3	1/5	2/4	2/4	1/2	1/1	1,138,479	472,106	195,773
AML22	Relapse	0/3	1/3	4/5	2/2	2/4	3/3	2/2	----	91,882	38,300	15,965

Frequency is one LSC per n cells. *The lower and upper frequencies were calculated at 95% Confidence Interval.

Figure 2.1. Identification of the frequencies of CD34 and CD38 expression populations in normal BM and AML bulk cells

(A) Representative flow cytometric profiles of CD34 and CD38 in normal bone marrow cells (NBM) (n = 3). (B) Representative flow cytometric profiles of CD34 and CD38 in AML bone marrow or peripheral blood cells at diagnosis (left panel) and relapse (right panel) (n = 3; AML 1, AML 3, AML 22). (C) Flow cytometric profiles of CD34 and CD38 in leukemic bone marrow or peripheral blood cells at diagnosis (left panel) and relapse (right panel) for 25 pairs of AML patients.

(A)

NBM4

NBM5

NBM6

(B)

AML1

AML3

AML22

Figure 2.2. Results of leukemic engraftment in xenotransplantations from phenotypic distinct fractions of 7 pairs of AML patient samples at diagnosis and relapse

(A) Engraftment levels of sorted cells of four populations ($CD34^+CD38^-$, $CD34^+CD38^+$, $CD34^-CD38^+$, $CD34^-CD38^-$) as well as unsorted cells from a representative AML sample (AML3) at diagnosis and relapse. Each dot represents the human CD45 positive engraftment level in BM of individual NSG mouse and the percentage greater than 0.1 is defined as leukemic engraftment. The horizontal line is the mean of engraftment level in NSG mice of each population. (B) Summary of the LSC activity in seven sorted AML samples (as defined by $CD34$ and $CD38$ or $CD32$ and $CD38$) at diagnosis and relapse. The equivalent dose of mononuclear cells (MNCs) per mouse is 5×10^5 to 3×10^6 cells for sorted populations. Plus sign indicates LSC detected at this sensitivity; minus sign indicates no LSC detected at this sensitivity. *LSC activity is detected using NSG-SGM3 (NSGS) mouse due to limited materials. (C) Engraftment levels of sorted cells of four populations ($CD34^+CD38^-$, $CD34^+CD38^+$, $CD34^-CD38^+$, $CD34^-CD38^-$ or $CD32^+CD38^-$, $CD32^+CD38^+$, $CD32^-CD38^+$, $CD32^-CD38^-$) as well as unsorted cells from a seven pairs of AML samples at diagnosis and relapse. Each dot represents the human CD45 positive engraftment level in BM of individual NSG mouse and the percentage greater than 0.1 is defined as leukemic engraftment. The horizontal line is the mean of engraftment level in NSG mice of each population. The equivalent dose of mononuclear cells (MNCs) per mouse is 5×10^5 to 3×10^6 cells for sorted populations. Unsorted group is injected with 1×10^6 cells per mouse. *LSC activity is detected using NSGS mouse due to limited

materials. (D) CD32 and CD38 expression profiles for 2 pairs of AML patient samples at diagnosis and relapse (AML18 and AML25).

(A)

(B)

AML	Status	CD34 ⁺ CD38 ⁻	CD34 ⁺ CD38 ⁺	CD34 ⁻ CD38 ⁺	CD34 ⁻ CD38 ⁻
AML1	Diagnosis	-	+	-	-
AML1	Relapse	+	+	-	-
AML2*	Diagnosis	+	+	-	-
AML2	Relapse	+	+	+	+
AML3	Diagnosis	+	+	-	-
AML3	Relapse	+	+	+	+
AML15	Diagnosis	-	+	-	-
AML15	Relapse	+	+	+	+
AML22	Diagnosis	+	-	+	+
AML22	Relapse	-	+	+	+

AML	Status	CD32 ⁺ CD38 ⁻	CD32 ⁺ CD38 ⁺	CD32 ⁻ CD38 ⁺	CD32 ⁻ CD38 ⁻
AML18	Diagnosis	-	-	+	+
AML18	Relapse	+	+	+	+
AML25	Diagnosis	-	+	+	-
AML25	Relapse	-	+	+	+

(C)

Diagnosis

Relapse

AML1

AML2

AML3

AML15

AML22

Diagnosis

Relapse

AML18

AML25

Figure 2.3. Distinct LSC populations contain long-term self-renewal ability and partially recapitulate AML phenotype in xenografts.

(A) Serial transplantation of a representative AML sample (AML3) at diagnosis and relapse. Whole BM from primary NSG recipients was transplanted into secondary NSG recipients. All phenotypically distinct LSC populations were capable of engrafting secondary recipients. (B) Results of human CD34 and CD38 expression on CD45⁺ cells in the bone marrow of primary recipients. AML cells in xenograft BM derived from each sorted population were able to partially develop CD34 and CD38 expression phenotype as in primary AML specimens.

(A)

(B)

Table 2.3. Results of secondary xenotransplantations

AML	Status	Fraction of 1 st BMT	Dose of hCD45 cells/mouse	Mice with AML/ mice transplanted
AML2*	Diagnosis	CD34 ⁺ CD38 ⁻	1.06E+05	0/4
AML2*	Diagnosis	CD34 ⁺ CD38 ⁺	1.68E+05	2/2
AML2	Relapse	CD34 ⁺ CD38 ⁻	4.76E+05	4/4
AML2	Relapse	CD34 ⁺ CD38 ⁺	8.80E+05	4/4
AML2	Relapse	CD34 ⁺ CD38 ⁺	8.85E+05	4/4
AML3	Diagnosis	CD34 ⁺ CD38 ⁻	1.44E+04	3/3
AML3	Diagnosis	CD34 ⁺ CD38 ⁺	1.32E+04	2/3
AML3	Relapse	CD34 ⁺ CD38 ⁻	8.13E+05	4/4
AML3	Relapse	CD34 ⁺ CD38 ⁺	7.07E+05	4/4
AML3	Relapse	CD34 ⁺ CD38 ⁺	2.13E+05	4/4
AML3	Relapse	CD34 ⁺ CD38 ⁻	6.19E+05	4/4
AML15	Diagnosis	CD34 ⁺ CD38 ⁺	1.17E+04	0/1
AML15	Relapse	CD34 ⁺ CD38 ⁻	9.29E+04	0/4
AML15	Relapse	CD34 ⁺ CD38 ⁺	3.24E+05	3/3
AML15	Relapse	CD34 ⁺ CD38 ⁺	8.96E+04	3/4
AML15	Relapse	CD34 ⁺ CD38 ⁻	1.01E+04	0/1
AML18	Diagnosis	CD32 ⁺ CD38 ⁺	6.92E+04	0/5
AML18	Diagnosis	CD32 ⁺ CD38 ⁻	1.84E+05	0/4
AML18	Relapse	CD32 ⁺ CD38 ⁻	6.80E+05	2/2
AML18	Relapse	CD32 ⁺ CD38 ⁺	7.14E+05	1/1
AML18	Relapse	CD32 ⁺ CD38 ⁺	1.55E+05	5/5
AML18	Relapse	CD32 ⁺ CD38 ⁻	6.13E+04	5/5
AML22	Diagnosis	CD34 ⁺ CD38 ⁻	2.08E+05	1/1
AML22	Diagnosis	CD34 ⁺ CD38 ⁺	3.18E+06	2/2
AML22	Diagnosis	CD34 ⁺ CD38 ⁻	6.09E+03	0/1
AML22	Relapse	CD34 ⁺ CD38 ⁺	5.96E+05	1/1
AML22	Relapse	CD34 ⁺ CD38 ⁺	8.66E+05	4/4
AML22	Relapse	CD34 ⁺ CD38 ⁻	2.47E+05	1/1
AML25	Diagnosis	CD32 ⁺ CD38 ⁺	4.37E+05	2/2
AML25	Diagnosis	CD32 ⁺ CD38 ⁺	4.02E+06	2/2
AML25	Relapse	CD32 ⁺ CD38 ⁺	8.91E+05	Died before harvest.
AML25	Relapse	CD32 ⁺ CD38 ⁺	2.18E+06	1/1
AML25	Relapse	CD32 ⁺ CD38 ⁻	8.76E+04	2/2

Figure 2.4. CD34 and CD38 expression on hCD45⁺ cells of 1st XBM derived from 7 pairs of AML patient samples at diagnosis and relapse

AML2**Diagnosis**

CD34+CD38+

1st XBM

CD34+CD38-

Relapse

CD34+CD38+

1st XBM

CD34+CD38-

CD34-CD38+

AML3**Diagnosis**

CD34+CD38+

CD34+CD38-

Relapse

CD34+CD38+

CD34+CD38-

CD34-CD38+

1st XBM **1st XBM**

AML15

Diagnosis

CD34+CD38+

1st XBM

Relapse

CD34+CD38+

1st XBM

CD34+CD38-

CD34-CD38+

CD34-CD38-

AML18**Diagnosis**

CD32-CD38+

CD32-CD38-

Relapse

CD32+CD38+

CD32+CD38-

CD32-CD38+

CD32-CD38-

1st XBM **1st XBM**

AML22**Diagnosis**

CD34+CD38-

1st XBM

CD34-CD38+

CD34-CD38-

Relapse

CD34+CD38+

1st XBM

CD34-CD38+

CD34-CD38-

AML25 Diagnosis

CD32+CD38+

CD32-CD38+

Relapse

CD32+CD38+*

CD32-CD38+

CD32-CD38-

1st XBM

1st XBM

* CD34/CD38 on
hCD97+ cells

Figure 2.5. Identification of the frequencies of alternative LSC marker expression populations in normal BM and AML bulk, CD34⁺CD38⁻ and CD34⁺CD38⁺ cells

(A) A representative example of LSC marker immunophenotyping strategy in AML bulk cells (AML3, diagnosis). The LSC panel includes CD123, CD33, CD97, CD32, CD47, CD96, TIM-3, CD45RA, and HLA-DR (upper panels). The frequencies of LSC marker positive populations in bulk AML cells at diagnosis (middle column) and relapse (right column) compared to NBM cells (left column) (NBM, n = 18 to 23; AML, n = 17 to 24) (lower panels). (B) A representative example of LSC marker immunophenotyping strategy in CD34⁺CD38⁻ populations (AML3, diagnosis) (upper panels). The frequencies of LSC marker positive populations in AML CD34⁺CD38⁻ cells at diagnosis (middle column) and in relapse (right column) compared to NBM CD34⁺CD38⁻ cells (left column) (NBM, n = 18 to 23; AML, n = 17 to 24) (lower panels). (C) A representative example of LSC marker immunophenotyping strategy in CD34⁺CD38⁺ populations (AML3, diagnosis) (upper panels). The frequencies of LSC marker positive populations in AML CD34⁺CD38⁺ cells at diagnosis (middle column) and in relapse (right column) compared to NBM CD34⁺CD38⁺ cells (left column) (NBM, n = 18 to 23; AML, n = 17 to 24) (lower panels).

Mean $\pm$ SEM values are plotted. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Bulk

(B)

CD34+CD38-

(C)

CD34+CD38+

Figure 2.6. Identification of the frequencies of LSC marker expression populations in normal BM and AML stem cell populations

(A) A representative example of LSC marker immunophenotyping strategy in functionally defined LSC populations (AML3, diagnosis). Boxes represent the LSC fractions functionally defined using xenotransplantation assay in this patient (upper panels). The frequencies of LSC marker positive populations in AML LSCs at diagnosis (middle column) and relapse (right column) compared to NBM HSCs (left column) (NBM, n = 18 to 23; AML, n = 12) (lower panels). (B) A representative example of LSC marker immunophenotyping strategy in functionally defined non-LSC populations (AML3, diagnosis). Boxes represent the non-LSC fractions functionally defined using xenotransplantation assay in this patient (upper panels). The frequencies of LSC marker positive populations in AML non-LSCs at diagnosis (middle column) and AML LSCs in relapse (right column) compared to NBM HSCs (left column) (NBM, n = 18 to 23; AML, n = 9) (lower panels).

NBM HSC = CD34⁺CD38⁻ fraction. Mean $\pm$ SEM values are plotted. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

LSC to LSC

(B)

Non-LSC to LSC

Figure 2.7. Molecular characterization of functionally defined LSC populations at diagnosis and relapse

(A) Heat map showing the normalized relative expression of LSC-related genes in normal versus AML stem cell populations at diagnosis and relapse. Sorted normal HSC and AML LSC populations were analyzed by TLDA-based analysis. Four normal HSC populations ($CD34^+CD38^-CD90^+Lin^-$) and twelve paired AML LSC populations at diagnosis and relapse from seven samples were shown in here. (B) Heat map showing the normalized relative expression of LSC-related genes in normal versus AML stem cell populations at diagnosis and relapse. LSC and HSC data is clustered using Pearson correlation metric with average linkage. (C) Heat map showing the fold change of top impacted genes more enriched or selected in relapse than at diagnosis in AML LSC populations (red = up-regulated in relapse, green = down-regulated in relapse). The two-tailed Wilcoxon matched-pairs signed rank test and paired Student's t-test is used for comparing the states described. $P < 0.05$ is considered as significant. (D) Up- and down-regulated LSC-related genes between diagnosis to relapse generated from TLDA-based analysis. Normalized relative expression of LSC-related genes in normal versus AML stem cell populations at diagnosis and relapse were shown in here. Expression levels are normalized to universal human reference RNA (UHRR) and the average of reference genes *GAPDH* and *HPRT1*. The two-tailed Mann–Whitney test (NBM vs. AML) or Wilcoxon matched-pairs signed rank test (diagnosis vs. relapse) is used for comparing the states described. Mean $\pm$ SEM values are plotted. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. # *BCL6* is not in Majeti et. al. or Epprt et. al.

(A)

(B)

(C)

Paired diagnosis vs. relapse specimens

(D)

Up-regulated genes **Down-regulated genes**

- AML1 CD34+CD38+
- AML2 CD34+CD38-
- AML2 CD34+CD38+
- AML3 CD34+CD38-
- AML3 CD34+CD38+
- AML15 CD34+CD38+
- AML18 CD32-CD38+
- AML18 CD32-CD38-
- AML22 CD34-CD38+
- AML22 CD34-CD38-
- AML25 CD32+CD38+
- AML25 CD32-CD38+

Chapter 3

Characterization of The Role of IL1RAP in Acute Myelogenous Leukemia Stem Cell Properties Following Treatment and Progression

3.1 Abstract

Background

Interleukin-1 receptor accessory protein (IL1RAP) is a cell surface co-receptor molecule recently found to be up-regulated on primitive progenitor cells in chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML). Recent evidence suggests *IL1RAP* overexpression is correlated with poor clinical outcome in AML patients with normal karyotype. Moreover, anti-IL1RAP antibodies induce selective-killing in leukemic cells through antibody-dependent cell-mediated cytotoxicity (ADCC). At present, little is known about the functional role of IL1RAP in AML LSC populations during disease progression between diagnosis, remission and relapse.

Methods

Primary bone marrow aspirates and peripheral blood samples were collected from patients undergoing induction therapy for AML at diagnosis and the time of relapse. Functional identification and molecular signature characterization of LSC populations was performed in Chapter 2. IL1RAP was identified as being up-regulated in LSC populations at relapse in the majority of LSC populations. We examined the protein expression level of IL1RAP on bulk and functionally defined LSC populations in paired pre-therapy and relapsed samples using flow cytometry. Lentiviral shRNA knockdown studies in primary AML cells and leukemia U937 cell line were performed to determine the functional role of IL1RAP in AML. Drug treatment and RNA-seq were performed to preliminary identify potential downstream mechanisms of IL1RAP in AML.

Results

Using flow cytometry with primary paired-AML specimens, we confirmed that IL1RAP is overexpressed on the surface of bulk, CD34⁺CD38⁻ and CD34⁺CD38⁺ cells (n = 25) and functionally defined LSC populations (n = 12) at diagnosis and relapse compared to their counterparts in normal bone marrow. Lentiviral mediated knockdown of IL1RAP impaired cell survival and proliferation in primary AML as well as leukemic U937 cells. Interestingly, IL1RAP knockdown also reduced clonogenicity and engraftment growth of primary AML cells *in vitro* and *in vivo*. Preliminary mechanism studies demonstrate that blocking NF-κB signaling, a well-known downstream pathway of IL1RAP, increased cell death in primary bulk AML cells. Preliminary RNA-seq data support NF-κB activation in the functionally defined LSC populations.

Conclusions

Our study identifies a functional role of IL1RAP in AML pathogenesis and leukemic stem cell/progenitor function. We demonstrate that overexpression of IL1RAP on AML cells and functionally defined LSC populations during clinical courses. Loss of IL1RAP overexpression can induce cell death and reduce clonogenicity and engraftment potential in primary AML cells. Taken together, these data implicate IL1RAP in the pathogenesis of AML and support targeting IL1RAP to develop new AML regimens in future studies.

3.2 Introduction

IL-1 receptor accessory protein (IL1RAP) is a co-receptor of IL-1 receptor (IL-1R) and IL-33 receptor (ST2) and plays a key role in inflammatory responses (Dinarello, 2009). IL1RAP was recently reported its importance in the acute and chronic myelogeneous leukemia. It has been shown that IL1RAP is up-regulated on the surface of CD34⁺CD38⁻ cells in CML patients compared to NBM samples (Jaras et al., 2010). By generating a polyclonal anti-IL1RAP antibody, they demonstrated that the IL1RAP targeting antibody can kill CML CD34⁺CD38⁻ cells by inducing antibody-dependent cell mediated cytotoxicity (ADCC) without injuring their normal counterparts (Jaras et al., 2010). A similar effect was found in AML. In a recent study, overexpression of IL1RAP was found on the surface of leukemic progenitors from AML patients with complete loss or deletions of the long arm of chromosome 7 (-7/7q-) and of patients with high-risk myelodysplastic syndrome (MDS) (Barreyro et al., 2012). IL1RAP overexpression on the cell surface was also demonstrated in AML with variable genetic subtypes (Askmyr et al., 2013), suggesting that up-regulation of IL1RAP expression is a common feature of AML. A newly generated chimeric anti-IL1RAP monoclonal antibody was shown to selectively kill AML mononuclear cells (MNCs), CD34⁺CD38⁺ and CD34⁺CD38⁻ cells *ex vivo* through ADCC effect (Askmyr et al., 2013), which is in agreement with previous findings (Jaras et al., 2010). Notably, high gene expression level of IL1RAP is independently associated with poor overall survival in AML with normal karyotype (Barreyro et al., 2012). Interestingly, knockdown of IL1RAP in some AML cell lines leads to decreased clonogenicity and increased cell death *in vitro* (Barreyro et al., 2012).

These results implicate that IL1RAP is a potential therapeutic target to selectively target AML cells.

IL1RAP mediates the inflammatory response of IL-1 and IL-33, members of the IL-1 superfamily (Dinarello, 2009). There are two forms of IL-1, termed IL-1 α and IL-1 β , which bind to the IL-1R1/IL1RAP complex thereby promoting IL-1 signaling. IL-33 binds to the ST2/IL1RAP dimer and participates in regulating the T-cell mediated immune responses (Kakkar & Lee, 2008). Previous studies have shown that IL-1 is produced as an autocrine factor in AML (Cozzolino et al., 1989; Turzanski et al., 2004) and exogenous IL-1 treatment increases cell survival and proliferation of AML blast cells *in vitro* (Cozzolino et al., 1989; Turzanski et al., 2004). In contrast, blocking IL-1 signaling by IL-1 receptor antagonist (IL-1ra), soluble IL-1 receptors (sIL-1R) or IL-1 neutralizing antibodies (anti-IL-1 α and anti-IL-1 β) in AML cells inhibits cell proliferation *in vitro* (Bruserud, 1996; Estrov et al., 1992; Rambaldi et al., 1991). Interestingly, the bone marrow serum from AML patients has lower levels of IL-1ra protein than normal bone marrow serum (Tao et al., 2000), suggesting a lower inhibition of IL-1 signaling from microenvironment in AML.

In this chapter, we have identified overexpression of IL1RAP on the surface of AML bulk, CD34⁺CD38⁻, CD34⁺CD38⁺ and functionally defined LSC populations in the majority of AML specimens at diagnosis and relapse compared to their normal counterparts. Knockdown of IL1RAP in primary AML and leukemic U937 cells increased cell death while reducing cell proliferation and clonogenicity *in vitro* as well as engraftment potential *in vivo*, which suggests an important role of IL1RAP in AML

pathogenesis and stem cell/progenitor function. Our preliminary data of drug treatment and RNA-seq analyses suggest that NF- κ B is a potential mechanism underlying IL1RAP to mediate cell survival and maintain LSCs in AML during clinical course.

3.3 Materials and Methods

Patients

Twenty-five paired AML samples were obtained as previously described (Chapter 2). We also included five other AML specimens for functional assays. NBM CD34⁺ cells were enriched using MACS Indirect CD34 MicroBead Kit (Miltenyi Biotec 2014) according to manufacturer's instructions.

Immunophenotyping

Thawed primary AML cells were stained with mouse anti-human antibodies against CD34 (PE-Cy7, 8G12, BD BioSciences), CD38 (APC, HB7, BD BioSciences), and IL1RAP (PE, 89412, R&D Systems). 4', 6 diamidino-2-phenylindole (DAPI) (Invitrogen) were used to exclude non-viable cells. Flow cytometry was performed on a LSR II (BD Biosciences). Flow cytometry was performed on a LSR II (BD Biosciences). For each specimen 3×10^5 to 5×10^5 events were analyzed. Data analyses were conducted using FlowJo software for Mac OS X (TreeStar, Ashland, OR) to determine the median fluorescence intensity (MFI) of defined populations relative to live cell gate. Normal bone marrow samples were processed under the same procedures as control.

High titer lentiviral production

The lentiviral cloning, high titer lentiviral production and lentiviral titer determination were described previously (Ashton et al., 2012). Briefly, to generate lentiviral particles targeting IL1RAP, the short hairpin RNA (shRNA) oligonucleotides (sh-IL1RAP C:

GCATCAAGTGCCCACTCTTTG; sh-IL1RAP E: GCTAGTGGTGATTCTCATTGT) were constructed into pLKO.1-GFP vector. Each shRNA was designed using Invitrogen Block-it RNAi Designer to cover all six IL1RAP transcripts. The lentivector pLKO.1-GFP construct DNA, pPax2 (packaging plasmid) and pMD2.G (vesicular stomatitis virus-g envelop plasmid) were transfected into 293TN cells (System Bioscience) using TransIT[®]-LT1 Transfection Reagent (Mirus Bio LLC) according to manufacturer's instructions. Next day morning, the transfection medium was replaced by fresh 293TN medium (DMEM plus 10% HI-FBS and 1% Glutamax). Lentiviral particles in supernatant were harvested on the following two days and filtered through 0.45 μ m PES syringe filter (Nalgene). Pooled lentiviral supernatant were either directly aliquoted in cryovials (Corning) or PEG precipitated before being aliquoted, and then flash frozen using liquid nitrogen followed by storage at -80°C.

Infection of human primary cells and leukemic U937 cells

Primary AML or NBM CD34⁺ cells were cultured in IMDM (Cellgro) based serum free medium (SFM) with recombinant human (rh) SCF (25 ng/mL), rh IL-3 (10 ng/mL), rh IL-7 (10 ng/mL), rh FLT3 (10 ng/mL), and rh GCSF (10 ng/mL) for 5 to 6 hours before infection. 5×10^6 primary cells were resuspended in 500 μ L cytokine SFM and mixed with 50 μ L PEG-concentrated viral supernatant and 5 μ L polybrene. To infect human leukemic U937 cells, 400 μ L of non-concentrated viral supernatant and 8 μ L polybrene was added into 0.5×10^6 - 2×10^6 cells in 400 μ L RPMI medium plus 10% heat-inactivated FBS (HI-FBS) and 1% penicillin/streptomycin (Pen Strep) (Gibco). The

infection was carried out at 37°C and 5% CO₂ overnight. Fresh 500 µL cytokine SFM or 1 mL U937 medium was added to cells in the end of infection. The infection efficiency was evaluated using flow cytometry on a LSR II (BD Biosciences). Live GFP⁺ cells (Dapi⁻GFP⁺ gating) were sorted under sterile conditions 48 to 72 hours post infection using a FACS Aria cell sorter (BD Biosystems) for following assays. For xenograft studies and some colony forming assays (CFU), live T-cell-deplete GFP⁺ primary AML cells (Dapi⁻CD3⁻GFP⁺ gating) were sorted 72 hours post infection into 1x PBS plus 0.5% HI-FBS at appropriate concentration for further use. The purity of each sort was subsequently checked using same instrument to ensure sort quality. The knockdown efficiency was confirmed by qRT-PCR, flow cytometry or western blot analysis. For flow cytometry, 3 x 10⁴ - 5 x 10⁴ events were analyzed for each sample.

Cell count assay

Cells were seeded at the density of 1 x 10⁶ cells/mL in 96-well or 48-well plate. For IL1RAP knockdown experiment, sorted GFP⁺ primary AML cells were cultured in cytokine SFM and U937 cells were cultured in RPMI based U937 medium. Fresh medium was added or changed every two days. Cell number was determined by using Countess[®] Automated Cell Counter (Life Technologies) every other day.

Cell death assay/Apoptosis assay

1 x 10⁵ cells were stained with Annexin V (APC, BD Pharmingen) and 4', 6 diamidino-2-phenylindole (DAPI) (Invitrogen) or 7AAD (Invitrogen) in 1X Annexin V Binding

Buffer (10 mM HEPES plus 140 mM NaCl and 2.5 mM CaCl_2) for 15 minutes at room temperature in the dark. Analysis was performed by flow cytometry using a LSR II (BD Biosciences). 3×10^4 - 5×10^4 events were analyzed for each sample.

Cell proliferation assay

Cells were stained using CellTrace™ Violet Cell Proliferation Kit (Life Technologies) according to manufacturer's instructions. Stained cells were returned to culture conditions and analyzed using flow cytometry on a LSR II (BD Biosciences) on the day of staining and after 24 and 48 hours in culture. 5×10^4 events were analyzed for each sample.

Cell cycle assay

Cells were fixed using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) per manufacturer's instructions. Fixed cells were then stained with anti-Ki-67 antibody (Alexa Fluor® 647, B56, BD Pharmingen) for 45 minutes at 4°C followed by resuspension in 7AAD (Invitrogen) with overnight incubation at 4°C. Flow cytometry was performed on a LSR II (BD Biosciences) and analyzed using FlowJo software for Mac OS X (TreeStar, Ashland, OR). For each specimen, 5×10^4 events were analyzed.

Colony forming assay (CFU)

Cells were plated in MethoCult™ GF H4534 (STEMCELL Technologies) with Epo (3 u/mL), GCSF (10 ng/mL) plus 1X Pen Strep (Gibco) on 35 mm petri dish (Corning) at 1

$\times 10^3$ cells/mL for U937 cells, 5×10^3 cells/mL for primary NBM CD34⁺ cells and 1×10^4 cells/mL for primary AML samples. Colonies were scored 14 days of culture for all samples.

NSG xenotransplantation assay

For xenograft studies, 8- to 10-week old NOD/SCID/interleukin 2 receptor γ chain null (NOD/SCID/IL2 γ ^{-/-}, termed NSG) mice received sublethal dose of radiation (250 cGy) using a RadSource X-Ray irradiator 24 hours prior to transplantation. Two doses of 0.5 mg/g of human intravenous immune globulin (IVIG) were applied in recipient mice by intraperitoneal (IP) injection on the day of irradiation and on the day of transplantation. Sorted live T-cell-deplete GFP⁺ primary AML cells (Dapi⁻CD3⁻GFP⁺ gating) were injected into recipient mice via tail vein injection in a final volume of 0.2 ml of PBS plus 0.5% HI-FBS. Equal numbers of sorted cells of each population (IL1RAP shRNA knockdown or control groups) were transplanted into recipients. Sorted populations with fewer than one million cells per mouse were mixed with syngeneic NSG splenocytes for transplantation. Mice were sacrificed 12 weeks post transplantation or at anytime they appeared moribund, and the mouse bone marrow (BM) was analyzed by flow cytometry. Leukemic burden was defined by the presence of GFP⁺ cells in mouse bone marrow. In addition, recipient BM cells were also stained with antibodies specific to mouse CD45, human CD45, human CD19 and human CD97 to ascertain the tumor burden as previously described (Chapter 2).

Drug treatment

Primary AML or leukemia U937 cells were cultured in presence of chemotherapy drugs (Cytarabine, Daunorubicin) or IL-1 signaling inhibitors (NSC697923, IKK-2 inhibitor IV, Stattic, STA-21, SP600125, SB202190, U0126) at indicated concentrations. The sources of drug/inhibitor were as follows: Cytarabine (Hospira Worldwide, Inc.), Daunorubicin (Teva Parenteral Medicines, Inc.), NSC697923 (NCI COMPOUND LIBRARY), IKK-2 inhibitor IV (EMD Millipore), Stattic (EMD Millipore), STA-21 (Enzo), SP600125 (CALBIOCHEM), SB202190 (EMD Millipore), and U0126 (Promega). Primary AML cells were cultured in cytokine supplemented SFM and U937 cells were cultured in RPMI with 10% HI-FBS plus 1% Pen Strep. After 24 and 48 hours of incubation, cells were harvested and stained with Annexin V/DAPI to determine for cell viability by flow cytometry.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) or RNeasy Plus Micro Kit (Qiagen) according to the instructions of manufacturer. cDNA was synthesized with iScript™ cDNA synthesis Kit (Bio-Rad). For individual gene, qRT-PCR was performed using TaqMan® Gene Expression Assay, TaqMan® PreAmp Master Mix and TaqMan® Gene Expression Master Mix (Life Technologies, Applied Biosystems) and carried out on the LightCycler® 480 Real-Time PCR System (Roche). The relative quantification analysis was performed using *GAPDH* and *HPRT1* as reference controls.

Western blot analysis

Primary antibodies for IL1RAP and GAPDH were purchased from Santa Cruz. HRP-conjugated secondary antibodies were purchased from Bio-Rad. Chemiluminescence detection was imaged using the ChemiDoc™ MP System with Image Lab™ Software (Bio-Rad).

Deep sequencing (RNA-seq) analysis of primary AML and NBM cells

For deep sequencing analysis, NBM HSC and AML functionally defined LSC or non-LSC populations were double-sorted in RLT Plus buffer (Qiagen) with beta-Mercaptoethanol (Sigma-Aldrich). Total RNA was extracted using the AllPrep DNA/RNA Micro Kit (Qiagen) following the protocols of manufacturer. RNA concentration was evaluated using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and the quality was further determined using Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA sequencing was performed by UR Genomics Research Center. Briefly, the library was prepared by using TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina) and analyzed by HiSeq2500 (Illumina).

3.4 Results

IL1RAP is overexpressed in AML at diagnosis and in relapse

We previously identified *IL1RAP* as up-regulated in functionally defined LSC populations between diagnosis to relapse in the majority of AML patients (Chapter 2). Recent reports have demonstrated the overexpression of IL1RAP on the surface of AML and CML cells compared to normal bone marrow (BM) (Askmyr et al., 2013; Barreyro et al., 2012; Jaras et al., 2010). However, it is unknown whether overexpression of IL1RAP is maintained on the cell surface of AML cells following treatment and disease progression. To address this question, we first examined the expression of IL1RAP on 25 paired diagnostic and relapsed AML primary bone marrow (BM) or peripheral blood (PB) specimens (Table 2.1, Chapter 2). Cells were stained with an anti-human IL1RAP antibody and analyzed by flow cytometry. Similar to previous reports (Askmyr et al., 2013; Barreyro et al., 2012), we found an increase in cell surface staining of IL1RAP in bulk cells from the majority of AML patients tested compared to normal BM samples. The IL1RAP expression in normal BM samples is low. More importantly, IL1RAP was consistently increased in AML patients at diagnosis and relapse (Figure 3.1A). Although recent studies and our work have demonstrated that LSC populations were not restricted to CD34⁺CD38⁻ population as previously described (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2008; Taussig et al., 2010), CD34⁺CD38⁻ and CD34⁺CD38⁺ populations contain primitive and committed progenitor cells in normal BM. Therefore, we next examined IL1RAP expression in AML CD34⁺CD38⁻ and CD34⁺CD38⁺ populations at diagnosis and relapse in comparison to their normal counterparts (Figures

3.1B and 3.1C). We found that IL1RAP was also able to distinguish leukemic from normal BM cells in $CD34^+CD38^-$ and $CD34^+CD38^+$ populations. Up-regulation of IL1RAP in $CD34^+CD38^-$ and $CD34^+CD38^+$ populations was stably expressed in the majority of AML specimens between diagnosis to relapse.

Prior studies of IL1RAP in AML did not functionally assess LSC populations or the impact of chemotherapy and disease progression. We analyzed IL1RAP expression on 12 pairs of functionally defined LSC populations from 7 AML patients at diagnosis and relapse identified by NSG xenotransplantation in Chapter 2 (Figure 3.2A). We demonstrated for the first time that IL1RAP is highly expressed on the surface of functionally defined LSC populations in pre-therapy and relapsed samples in comparison with NBM HSC populations ($CD34^+CD38^-$ cells) (NBM HSCs = 1.7 ± 1.1 , diagnostic AML LSCs = 3.2 to 49.9, relapsed AML LSCs = 3.8 to 85.7) (Figure 3.2A). We then examined 12 pairs of functionally defined non-LSC populations at diagnosis that generated LSC activity in relapse from 7 AML patients (Figure 3.2B). Interestingly, overexpression of IL1RAP was also found in functionally defined non-LSC populations at diagnosis in the majority of AML patients and showed a trend of up-regulation from diagnosis to relapse. To examine the expression levels of key genes in IL-1/IL1RAP pathway and IL-33/IL1RAP pathway in functionally defined LSC populations at diagnosis and in relapse, RNA-seq analyses were performed on 9 pairs of functionally defined LSC populations from 6 AML patients identified by NSG xenotransplantation assays (Chapter 2) and compared to normal HSC populations ($CD34^+CD38^-CD90^{low}Lin^-$). We demonstrated that the expression levels of the IL-1 pathway (Figure 3.3A) genes as

well as the IL-33 pathway genes (Figure 3.3B) are similar between HSC and LSC populations at diagnosis and relapse. The expression level of *IL1A* (IL-1 α) in functionally defined LSC populations is lower than NBM HSCs; however, the expression levels are generally low in both HSC and LSC populations.

IL1RAP expression is important to cell survival and LSC function in AML

Given that IL1RAP is overexpressed in AML at diagnosis and relapse, we investigated whether IL1RAP is functionally relevant to leukemic stem and progenitor populations. We targeted the *IL1RAP* gene in primary AML and U937 cells, a myelomonocytic leukemia cell line, by two independent shRNA lentiviruses as detailed above. At 48 to 72 hours post infection, live GFP⁺ cells were sorted and assessed for cell proliferation, cell cycle, apoptosis, colony forming and stem cell activity by xenotransplantation. Both shRNA constructs employed in our knockdown studies demonstrated efficient reduction of IL1RAP RNA and protein levels (Figure 3.4).

Knockdown of IL1RAP impaired proliferation as assessed by cell counts (Figure 3.5A). To confirm the impact of IL1RAP knockdown on cell proliferation, sorted GFP⁺ U937 cells 3 days post infection were labeled with CellTrace™ Violet reagent and live GFP⁺ populations were analyzed using flow cytometry at 24 and 48 hours. CellTrace™ Violet reagent diffuses into cells and is cleaved by intracellular esterases turning the reagent into a fluorescent compound that covalently binds to intracellular amines. Upon cell proliferation, the CellTrace™ Violet dye is equally divided between daughter cells and the median fluorescence intensity (MFI) of dye is reduced. At 24 and 48 hours after

staining, we found the MFI of dye in IL1RAP knockdown groups decreased slower than control groups, which is consistent with a reduction in cell proliferation (Figure 3.5B). However, cell cycle analysis at 4 days post infection showed no significant difference between the IL1RAP knockdown groups and control groups in primary AML and U937 cells, suggesting the cell cycle state is not affected at this time point by the down-regulation of IL1RAP (Figure 3.5C). Alternatively, the increase in cell apoptosis in the knockdown limits the ability to interrogate the dataset at this timepoint. A recent study showed that loss of IL1RAP induced cell death in THP-1 cells, an AML cell line with high expression level of IL1RAP (Barreyro et al., 2012). We performed an Annexin V apoptosis assay on sorted GFP⁺ cells. Knockdown of IL1RAP led to a decrease in cell viability and an increase in the frequency of apoptotic and dead cells compared to control groups. These effects were greatest at 5 days post infection (Figure 3.5D).

To further evaluate the relevance of IL1RAP in leukemia stem cell populations, we performed *in vitro* colony forming assays on primary AML and U937 cells with or without IL1RAP knockdown. Sorted GFP⁺ cells at 3 days post infection were plated in methylcellulose-based matrix and scored the colony forming units (CFU) after 14 days of culture. In four primary AML samples, the leukemic cells with IL1RAP knockdown significantly reduced the colony forming ability in comparison to control groups (Figure 3.6A). Similar results were seen using U937 cells (Figure 3.6A). These results demonstrate that inhibition of IL1RAP alters clonogenicity in primary AML cells and in U937 cells. To functionally assess the long-term effect of down-regulation of IL1RAP in LSCs, xenograft transplantation assays were performed using NOD/SCID/IL2r $\gamma^{-/-}$ (NSG)

mice. Primary GFP⁺ AML cells were sorted 3 days post infection and transplanted into NSG mice. Equal numbers of sorted cells with or without IL1RAP knockdown were transplanted into recipient NSG mice and engraftment levels in mouse bone marrow were determined after 12-13 weeks post transplantation or at anytime they appeared moribund. As shown in Figure 3.6B, LSC activity was impaired when IL1RAP expression was lost in three separate primary AML samples. Due to material limitations, we transplanted two samples with less than one million cells (1.5×10^5 and 1×10^5 , respectively) into NSG mice contributing to relatively lower engraftment levels. In addition, scramble shRNA showed some effects on reduction of leukemia cells in mouse BM, but overall observation showed that knockdown of IL1RAP generally decreased leukemia burden in recipient mice, indicating the important functional role of IL1RAP in stem cell populations in primary AML.

We demonstrate that the expression levels of IL1RAP are generally lower in normal CD34⁺CD38⁻ and CD34⁺CD38⁺ populations (Figure 3.1). To assess the impact of loss of IL1RAP in the context of normal hematopoiesis, we performed knockdown of IL1RAP in enriched CD34⁺ cells from normal donors. In humans, CD34⁺ cells contain primitive and committed progenitor cells. We enriched NBM CD34⁺ cells by using MACS Indirect CD34 MicroBead Kit, and infected these cells with lenti-viruses. Live GFP⁺CD34⁺ cells were sorted 3 days post infection and plated in methylcellulose-based matrix for *in vitro* colony forming assays. After 14 days of culture, we found the colony forming ability of IL1RAP knockdown groups dropped approximately 60% compared to control groups, unlike the dramatically diminished clonogenicity found in primary AML

and U937 cells with IL1RAP knockdown. These results suggest that IL1RAP is less essential for human normal HSCs or progenitor cells than AML LSCs (Figure 3.6C).

Taken together, our findings indicate that IL1RAP is indispensable to mediate cell survival in AML cells. Loss of IL1RAP dramatically impairs the short-term clonogenic capacity in AML and the stem cell ability to engraft in recipients, but has fewer effects on normal HSCs or progenitor cells. It suggests that overexpression of IL1RAP may contribute to leukemogenesis and promote survival of leukemic stem and progenitor cells, which can be a good target for developing novel therapeutics.

IL1RAP is not directly related to the resistance of leukemic cells to conventional chemotherapy drugs for AML

Our findings that the expression of *IL1RAP* increases between diagnosis and relapse suggest a potential role for IL1RAP in AML cells in resistance to conventional chemotherapy. To test this possibility, we evaluated the sensitivity of leukemic cells with or without IL1RAP knockdown to conventional chemotherapy drugs for AML, cytarabine (Ara-C) and anthracycline (eg. Daunorubicin, DNR). We first treated U937 cells *in vitro* with various concentrations of Ara-C and DNR to titrate their resistance to chemotherapy. After 24 and 48 hours of treatment, cell viability was measured with Annexin V/viability dye using flow cytometry. As shown in Figure 3.7A, 5 μ M of Ara-C or 250 nM of DNR can induce ~50% of toxicity. U937 cells are minimally affected by Ara-C with up to 15 μ M and prolonged treatment times (48 hours) minimally impacting cell viability. We then assessed the impact of IL1RAP knockdown on response to Ara-C

and DNR. Given that decreased expression of IL1RAP generally induced a significant cell death after 5 days post infection, we performed the drug treatment at 3 or 4 days post infection. IL1RAP knockdown had no impact on chemotherapy induced cell death after 24 or 48 hours of treatment (Figures 3.7B and 3.7C). Taken together, our data suggest that IL1RAP may not be directly related to drug resistance of AML cells in the short run but may sustain AML cells to survive after chemotherapy in the long run.

The role of IL1RAP in AML pathogenesis may appear to function through NF- κ B pathway rather than MAPK/c-Jun pathway

Given that knockdown of IL1RAP can dramatically induce AML cell death and impair LSC ability in culture and in xenotransplantation assays, characterizing IL1RAP signaling in AML LSC populations may shed light on critical survival mechanisms in this population. NF- κ B and MAPK/c-Jun pathways are two well-known downstream pathways of IL-1/IL1RAP signaling. We first assessed the role of these pathways in LSC survival using a chemical inhibitor screen. Bulk BM/pheresis cells from five AML patients were treated with increasing concentrations of multiple known inhibitors against different components in NF- κ B and MAPK/c-Jun pathways. After 24 and 48 hours, cells were harvested and cell viability/apoptosis analyzed by flow cytometry (Figure 3.8). Ubc13, a downstream component of IL-1 signaling, is known as an E2 enzyme conjugated with E3 enzyme TRAF6 to activate both NF- κ B and MAPK/c-Jun pathways (Kasza, 2013). After 48 hours of treatment, we found the Ubc13 inhibitor NSC697923 induced significant cell death in the majority of primary bulk AML specimens (Figure

3.8A). Similarly, an IKK-2 inhibitor IV, which leads to blocking of NF- κ B signaling, also induced ~40 to 70% toxicity in AML specimens in response to 20 μ M IKK-2 inhibitor IV after 48 hours of incubation (Figure 3.8B). This observation is consistent with previous studies suggesting that inhibition of NF- κ B pathway with MG-132 proteasome inhibitor elicited a potent apoptosis in total and CD34⁺CD38⁻ AML cells by preventing the degradation of negative regulator I κ B α (Guzman et al., 2001). In addition, previous references have reported that STAT3 is an important modulator of NF- κ B (Kasza, 2013). Unphosphorylated STAT3 competes with I κ B for the binding of NF- κ B in the cytoplasm and assists the nuclear localization of NF- κ B. STAT3 can also recruit p300 histone acetylase to acetylate NF- κ B in the nucleus, preventing binding by I κ B and prolonging its nuclear localization. We also assessed the effect of two distinct STAT3 inhibitors, Stattic and STA-21, in primary AML bulk cells. STAT3 inhibitors similarly induced cell death in AML cells (Figures 3.8C and 3.8D). In contrast, inhibition of MAPK/c-Jun pathway, using JNK inhibitor SP600125, p38 inhibitor SB202190 and MEK1/2 inhibitor U0126, showed no significant effect on primary AML cells after 24 and 48 hours of treatment (Figures 3.8E-G). Taken together, these findings confirm that NF- κ B pathway signaling has an essential role in regulation of cell survival in AML cells. The downstream mechanism of IL1RAP in leukemogenesis and promoting survival of leukemic cells may occur via the NF- κ B pathway.

Constitutive activation of the NF- κ B pathway has been reported in primary human AML CD34⁺, CD34⁺CD38⁻ and CD34⁺CD38⁻CD123⁺ populations (Guzman et al., 2001; Kagoya et al., 2014) as well as leukemia-initiating cells (LICs) from different

types of mouse myeloid leukemia models (Kagoya et al., 2014), but not in normal hematopoietic cells (Guzman et al., 2001; Kagoya et al., 2014). To test our hypothesis that NF- κ B pathway is the potential downstream mechanism of IL1RAP regulating cell survival and maintain LSCs in AML during clinical course, we further characterized NF- κ B activity in functionally defined LSC populations. We examined the gene expression levels of key genes in the NF- κ B pathway in functionally defined LSC populations at diagnosis and in relapse. RNA-seq analyses were performed on 9 pairs of functionally defined LSC populations from 6 AML patients identified by NSG xenotransplantation assays (Chapter 2) and compared to normal HSC populations ($CD34^+CD38^-CD90^{low}Lin^-$). The gene expression levels of NF- κ B pathway are shown in Figure 3.9A. Expression levels of several NF- κ B pathway genes including *NFKB1* (p50), *RELA* (p65), *NFKBIA* (I κ B- α), *NFKBIB* (I κ B- β) and *IKBKB* (IKK- β) were included in the analysis. Kagoya et al. examined levels of total p65 in normal GMP and LIC populations in three mouse myeloid leukemia models and identified no significant difference (Kagoya et al., 2014). We found lower levels of expression of *RELA* (p65) in functionally defined LSC populations at diagnosis and relapse in comparison to normal HSC. We also investigated the expression levels of a subset of genes known to be regulated by NF- κ B. The RNA-seq analyses showed that five NF- κ B target genes (*BCL2*, *ITGB5*, *LITAF*, *PLAU*, and *TNF*) were up-regulated in functionally defined LSC populations in pre-therapy and relapsed samples compared to normal HSCs (Figure 3.9B). Currently the sample size of normal HSC group is too small to perform statistical analysis. More normal HSC samples are needed for our future work to validate these findings. We further examined the impact of

IL1RAP knockdown on NF- κ B activation in primary AML and U937 cells using qPCR. We found knockdown of IL1RAP decreased the expression levels of TNF- α , which is a NF- κ B target gene, in three out of five primary AML samples. Similar results were found using U937 cells (Figure 3.10). These data indicate that the NF- κ B pathway may be activated in primary human AML LSC populations at diagnosis and in relapse. It also suggests that NF- κ B pathway is a potential mechanism underlying IL1RAP to regulate AML pathogenesis and LSC functions. Validation studies are underway.

3.5 Discussion

We previously examined the change of LSC signaling during patients' clinical course in functionally defined LSC populations (Chapter 2). When assessing the change of individual genes within the majority of patients, IL1RAP, was significantly up-regulated between diagnosis and relapse. We hypothesize that IL1RAP plays an important role in AML pathogenesis and therefore functionally tested our hypothesis in this chapter. While overexpression of IL1RAP has been reported for AML and CML cells (Askmyr et al., 2013; Barreyro et al., 2012; Jaras et al., 2010), little is known about IL1RAP expression on LSC populations and the impact of therapy and subsequent relapse on its expression. We investigated the expression of IL1RAP on the surface of twenty-five paired bulk populations and twelve paired functionally defined LSC populations in pre-therapy and relapsed AML samples. We demonstrate the overexpression of IL1RAP on bulk, CD34⁺CD38⁻, CD34⁺CD38⁺ as well as functionally defined LSC populations in the majority of AML patients at diagnosis and in relapse compared to NBM. For the first time we show that IL1RAP expression was not diminished on primary AML samples following drug treatment and disease progression and thus may be useful for assessment of MRD as well as represent a therapeutic target for novel AML treatment regimens. However, while the expression of the IL1RAP gene was up-regulated following relapse in functionally defined LSC populations in the majority of the AML samples (Chapter 2), the level of surface protein of IL1RAP did not show a significant change for functionally defined LSC populations between diagnosis to relapse. Similarly, based on direct comparison within individual patients during clinical course, the cell surface IL1RAP

expression was maintained but not up-regulated in AML bulk, CD34⁺CD38⁻ and CD34⁺CD38⁺ cells. In bulk AML cells, 40% of patients are up-regulated, 40% are similar and 20% were decreased. IL1RAP has six different transcripts encoding both membrane-bound as well as soluble isoforms (NCBI). The TaqMan[®] probe used for qRT-PCR cover all six transcripts. For assessment of protein levels, we relied on flow cytometry which assessed only the membrane-bound isoform. To validate the total protein expression level of IL1RAP (membrane-bound or soluble isoforms) from diagnosis to relapse, future work will need to analyze the total cell lysates from paired bulk and functionally defined LSC populations using western blot analysis.

By performing IL1RAP knockdown with a lentiviral system, we demonstrated knockdown of IL1RAP expression impaired cell survival and proliferation *in vitro* in primary AML and U937 cells. We also showed that IL1RAP knockdown significantly reduced the clonogenicity *in vitro* and engraftment potential *in vivo*, suggesting a severe impairment in the progenitor and stem cell capacity in primary AML and U937 cells. Given that IL1RAP is functionally critical for AML cells, we currently are working on identifying molecular mechanisms underlying IL1RAP's role in AML LSCs. Our preliminary data demonstrated inhibition of NF-κB/STAT3 signaling (e.g., targeting IKK-2 and STAT-3) induced severe cell death in AML bulk cells. In addition, using RNA-seq analyses, we found an up-regulation of NF-κB target genes in functionally defined LSC populations in comparisons to NBM HSCs. IL-1 binding to its receptor is a known potent stimulator of NF-κB signaling.

Our findings demonstrate the functional importance of IL1RAP in AML

pathogenesis. Previous studies have shown that knockdown of IL1RAP increased cell death in leukemic THP-1 cells and decreased clonogenic potential in some AML cell lines (THP-1, OCI-AML3, HEL and HL-60) (Barreyro et al., 2012). A recent study reported the treatment of monoclonal antibody against IL1RAP (Mab81.2) *in vivo* reduced the leukemic MA9Ras cells in a xenograft NOD/SCID mouse model of AML (Agerstam et al., 2013) (ASH abstract). The leukemic MA9Ras cells were generated from human cord blood CD34⁺ cells transformed with MLL/AF9 fusion and an activated NRAS gene (Agerstam et al., 2013) (ASH abstract). However, they did not address whether the observed effect was through ADCC as in their previous report (Askmyr et al., 2013) or by direct inhibition of IL-1/IL1RAP signaling. Most of the above studies were performed by using leukemia cell lines or transformed leukemia cells, which may not reflect the biology in primary AML patient samples. Extending previous findings, our study further demonstrated that IL1RAP expression is important to cell growth, cell proliferation and cell survival in primary AML or U937 cells. We also demonstrated that knockdown of IL1RAP dramatically decreased the clonogenicity of primary AML and U937 cells *in vitro* as well as the engraftment ability of primary AML cells in recipient NSG mice *in vivo*, suggesting IL1RAP is an important regulator in AML stem and progenitor populations. Since we found IL1RAP overexpression in the functionally defined LSC populations in pre-therapy and relapsed AML specimens, our findings regarding the functional role of IL1RAP in AML point out a potential new strategy to eradicate LSC populations in AML patients during the clinical course.

Given that the targeting of IL1RAP may be a promising therapy of AML, we

further investigated whether knockdown of IL1RAP affects normal hematopoietic stem cell or progenitor ability. While knockdown of IL1RAP significantly reduced the CFU forming in primary AML and leukemia U937 cells, we demonstrated that IL1RAP knockdown showed a less dramatic effect on NBM CD34⁺ cells (~60% inhibition of CFU activity). IL1RAP knockout mice have been reported to be grossly healthy at birth, viable and fertile (The Jackson Laboratory), and mice deficient in IL-1 α , IL-1 β and IL-1R1 develop normally as well (Dinarello, 2009). A recent report investigated normal hematopoiesis in IL1RAP knockout mice (IL1RAP^{-/-}) and found a reduction of myeloid cells in peripheral blood (Hansen et al., 2013) (ASH abstract). By using competitive stem cell transplantations, they demonstrated that IL1RAP^{-/-} HSCs contributed similarly to IL1RAP^{+/+} HSC engraftment in primary and secondary recipients (Hansen et al., 2013) (ASH abstract), suggesting IL1RAP is involved in steady-state myelopoiesis. Using a monoclonal anti-IL1RAP antibody, it has been shown that there is a selective killing of AML CD34⁺CD38⁻ cells through ADCC, but NBM cells are spared (Askmyr et al., 2013). Overall, our findings and that of others demonstrate targeting IL1RAP is relatively innocuous to normal HSCs, suggesting an applicable approach to preferentially ablate LSCs in AML.

Current standard chemotherapy drugs for AML, such as cytarabine (Ara-C) and anthracyclines (eg. Daunorubicin), generally target cycling cells through interfering with DNA replication and inducing cell death. AML LSC population has been reported to be mostly quiescent and likely to escape from chemotherapy and contributes to relapse. Since standard chemotherapy may not target LSC populations, a new approach to target

LSC populations is an important priority. Given that overexpression of cell surface IL1RAP has been demonstrated in pre-therapy and relapsed specimens, we hypothesized that IL1RAP may be a mediator of drug resistance of AML LSCs following chemotherapy and disease progression. However, we did not observe any impact of the loss of IL1RAP expression following 24 or 48 hours of drug treatment (Ara-C or DNR). One possible explanation for this observation may be that leukemia cells transduced with IL1RAP shRNA demonstrate significant spontaneous apoptosis at 5 days post infection and significantly reduced colony forming ability. Thus, even we use cells at 3 or 4 days post infection for drug treatment assay, it is still likely that these leukemia cells with IL1RAP knockdown are already destined to undergo apoptosis. For future analysis, it will be important to knock-in IL1RAP and investigate whether overexpression of IL1RAP can increase the drug resistance in AML LSC populations that demonstrate sensitivity to these agents.

The possible downstream mechanism by which IL1RAP contributes to the pathogenic potential of LSCs has not been well studied in previous reports. In our study, we treated primary AML specimens with inhibitors of multiple pathways previously reported to be mediated by IL1RAP, including IL-1 pathway, NF- κ B pathway, MAPK/c-Jun pathway, and we demonstrated that blocking of NF- κ B pathway induced cell death in the majority of AML bulk cells. We further demonstrated NF- κ B signaling is up-regulated in functionally defined LSC populations of primary AML patients at diagnosis and in relapse. Thus, our findings suggest the role of IL1RAP in leukemogenesis may function through the NF- κ B pathway. NF- κ B is a transcription factor that regulates genes

related to the cell survival, cell proliferation, cell adhesion, inflammation, immune responses and cell stress responses (Perkins, 2007). NF- κ B signaling is found constitutively activated in a variety of malignancies and it has been reported to play an important role in cancer development and progression (Karin & Greten, 2005). In addition, constitutive activation of NF- κ B has been found in primary AML cells (CD34⁺, CD34⁺CD38⁻ and CD34⁺CD38⁻CD123⁺), but not in normal hematopoietic cells (Guzman et al., 2001; Kagoya et al., 2014). A recent study using three myeloid leukemia mouse models also demonstrated that NF- κ B signaling is constitutively activated in leukemia-initiating cells (LICs), but not normal hematopoietic stem cells or non-LIC populations in leukemic BM cells (Kagoya et al., 2014). They further identified that LIC capacity was promoted by a positive feedback loop between NF- κ B and TNF- α (Kagoya et al., 2014). Interestingly, we also found the gene expression of TNF- α was highly expressed in functionally defined LSC populations in pre-therapy and relapsed primary AML samples. Therefore, important work for future studies will be to functionally validate whether NF- κ B is the mechanism underlying IL1RAP's contribution to AML pathogenesis, and to determine whether the NF- κ B/TNF- α feedback loop is also involved in the regulation of IL1RAP in primary AML LSC populations. Since NF- κ B pathway integrates with many other mechanisms (Perkins, 2007), detailed study to understand the cell-signaling networks regulated by IL1RAP and NF- κ B will be very helpful in developing novel diagnostic and therapeutic applications for AML patients in clinical use. Moreover, further analysis will be needed to determine whether there are alternative or non-canonical pathways through which IL1RAP contributes to AML pathogenesis. A

combination of different inhibitors or drugs may increase the probability to effectively ablate LSC populations in AML.

Figure 3.1. Identification of the cell-surface IL-1 receptor accessory protein (IL1RAP) on bulk, CD34⁺CD38⁻ and CD34⁺CD38⁺ cells in AML samples at diagnosis and relapse compared to normal bone marrow (NBM) counterparts

(A) Median fluorescence intensity (MFI) ratio of IL1RAP relative to FMO control for bulk cells in NBM, diagnostic and relapsed AML samples. (B) MFI ratio of IL1RAP relative to FMO control for CD34⁺CD38⁻ cells in NBM, diagnostic and relapsed AML samples. (C) MFI ratio of IL1RAP relative to FMO control for CD34⁺CD38⁺ cells in NBM, diagnostic and relapsed AML samples. MFI ratio = (Sample MFI – FMO MFI)/FMO MFI. NBM, n = 24; AML, n = 25. Mean ± SEM values are plotted. ****p < 0.0001.

Figure 3.2. Identification of cell-surface IL1RAP in functionally defined LSC populations from paired AML samples at diagnosis and relapse compared to NBM hematopoietic stem cells (HSCs)

(A) Median fluorescence intensity (MFI) ratio of IL1RAP relative to FMO control in functionally defined AML LSC populations at diagnosis (middle column) and in relapse (right column) compared to NBM HSCs (left column). (B) MFI ratio of IL1RAP relative to FMO control in functionally defined AML non-LSC populations at diagnosis (middle column) and LSC populations in relapse (right column) compared to NBM HSCs (left column). NBM HSC = CD34⁺CD38⁻ fraction. MFI ratio = (Sample MFI – FMO MFI)/FMO MFI. NBM, n = 24; AML, n = 12. Mean ± SEM values are plotted. ****p < 0.0001.

(A)

(B)

Figure 3.3. Expression levels of IL-1 and IL-33 pathway genes in functionally defined LSC populations at diagnosis and in relapse compared to NBM HSCs

Expression levels of (A) IL-1 pathway genes (B) IL-33 pathway genes in normal versus AML stem cell populations at diagnosis and in relapse generated from deep sequencing (RNA-seq) analyses. One normal HSC population (CD34⁺CD38⁻CD90⁺Lin⁻) and nine paired functionally defined LSC populations at diagnosis and relapse from six AML patients were shown in here. Mean $\pm$ SEM values are plotted. Wilcoxon matched-pairs signed rank test is used for comparing the diagnostic versus relapsed AML samples.

(A)

(B)

Figure 3.4. Identification of the expression levels of IL1RAP knockdown in leukemia cells

(A) IL1RAP mRNA expression in U937 cells transduced with empty vector, scramble shRNA or with two shRNA targeting IL1RAP (IL1RAP shRNA C and E) using lenti-viral system. qRT-PCR assays were performed using sorted GFP⁺ cells at 4 days post infection. Gene expression of IL1RAP was normalized to vector control group. Expression levels are normalized to universal human reference RNA (UHRR) and the average of reference genes *GAPDH* and *HPRT1*. n = 5 independent experiments. Technical triplicates were performed in each experiment. Mean $\pm$ SD values are plotted. The two-tailed Unpaired t-test is used for comparing the states described. ****p < 0.0001. (B) Median fluorescence intensity (MFI) ratio of IL1RAP relative to FMO control in GFP⁺ U937 cells. IL1RAP protein expression level was measured at 3, 4 and 5 days post infection using flow cytometry. n = 2 to 3 independent experiments. Mean $\pm$ SD values are plotted. (C) Western blot analysis in U937 cells. Sorted GFP⁺ cells were analyzed at 4 days post infection.

(A)

(B)

(C)

Figure 3.5. Knockdown of IL1RAP reduces cell survival and proliferation in primary AML cells or U937 cells.

(A) Cell count assay of primary AML cells and U937 cells with or without knockdown of IL1RAP. Sorted GFP⁺ cells were seeded at 1E6/mL at 4 days post infection, and counted every other day. (B) CellTrace™ Violet Cell Proliferation assay of U937 cells with or without knockdown of IL1RAP. Sorted GFP⁺ cells were stained with CellTrace™ Violet dye at 3 days post infection and returned to culture conditions. Stained cells were analyzed on the day of staining and after 24 and 48 hours in culture. Median fluorescence intensity (MFI) ratio of IL1RAP relative to FMO control, 0 hours baseline and vector control is plotted. (C) Ki-67 cell cycle assay of primary AML cells and U937 cells with or without knockdown of IL1RAP. Sorted GFP⁺ cells were fixed and then stained with anti-Ki-67 antibody and 7AAD at 4 days post infection. (D) Annexin V apoptosis assay of primary AML cells and U937 cells with or without knockdown of IL1RAP. 7AAD or DAPI were used as the DNA binding viability dyes. Sorted GFP⁺ cells were analyzed at 5 days post infection.

Data of U937 cells in figures (B) to (D) were generated from three independent experiments. Technical duplicates or triplicates were performed in each experiment. Mean ± SD values are plotted. The two-tailed Unpaired t-test is used for comparing the states described. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(A)

(B)

(C)

(D)

Figure 3.6. Knockdown of IL1RAP reduces clonogenicity and engraftment potential in primary AML cells or U937 cells.

(A) Colony forming ability of primary AML and U937 cells with or without knockdown of IL1RAP. Sorted GFP⁺ cells were plated at 3 days post infection, and CFU was counted at 14 days (except AML 1_R, plated at 4 days post infection). AML, n = 4; U937, n = 4 independent experiments. Technical triplicates were performed in each experiment. Mean $\pm$ SD values are plotted. The two-tailed Unpaired t-test is used for comparing the states described. ***p < 0.001, ****p < 0.0001. (B) Engraftment levels of primary AML samples with or without knockdown of IL1RAP. Each dot represents the GFP⁺ engraftment level in BM of individual NSG mouse analyzed by flow cytometry. The horizontal line is the mean of GFP⁺ engraftment level in NSG mice of each group. (C) Colony forming ability of NBM CD34⁺ cells with or without knockdown of IL1RAP. Sorted GFP⁺ cells were plated at 3 days post infection. (Upper panel) Pictures of CFU taken after 14 days of culture. (Lower panel) Quantification of CFU results. Technical triplicates were performed in each experiment. Mean $\pm$ SD values are plotted. The two-tailed Unpaired t-test is used for comparing the states described. ***p < 0.001

(A)

(B)

Primary cells

(C)

NBM CD34+

Figure 3.7. Knockdown of IL1RAP does not increase drug sensitivity in U937 cells.

(A) U937 cells were treated with Cytarabine (Ara-C) or Daunorubicin (DNR) for 24 and 48 hours. After incubation, cells were harvested and stained with Annexin V/DAPI to determine cell viability by flow cytometry. (B) U937 cells with or without knockdown of IL1RAP were treated with various concentrations of Ara-C and DNR for 24 and 48 hours and analyzed by flow cytometry to determine cell viability. Sorted GFP⁺ cells were seeded at 3 days post infection. Percentage of viable cells was normalized to untreated cells of each group. (C) U937 cells with or without knockdown of IL1RAP were treated with various concentrations of Ara-C and DNR for 24 hours and analyzed by flow cytometry to determine cell viability. Sorted GFP⁺ cells were seeded at 4 days post infection. Percentage of viable cells was normalized to untreated cells of each group.

Technical duplicates or triplicates were performed in each experiment. Mean $\pm$ SD values are plotted. The two-tailed Unpaired t-test is used for comparing the states described. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(A)

(B)

(C)

Figure 3.8. Inhibition of IL-1 and NF- κ B signaling induces cell death in primary AML cells.

Primary AML specimens were treated with various IL-1 signaling inhibitors for 24 and 48 hours. After incubation, cells were harvested and stained with Annexin V/DAPI to measure cell viability using flow cytometry. (A) Ubc13 inhibitor NSC697923, (B) IKK-2 inhibitor IV, (C) STAT3 inhibitor Stattic, (D) STAT3 inhibitor STA-21, (E) JNK inhibitor SP600125, (F) p38 inhibitor SB202190 and (G) MEK1/2 inhibitor U0126. Percentage of viable cells was normalized to vehicle group. AML, n = 4 to 5. Technical triplicates were performed in each experiment. Mean $\pm$ SD values are plotted.

Figure 3.9. Expression levels of NF- κ B pathway genes in functionally defined LSC populations at diagnosis and in relapse compared to NBM HSCs

Expression levels of (A) NF- κ B pathway genes (B) NF- κ B target genes in normal versus AML stem cell populations at diagnosis and in relapse generated from deep sequencing (RNA-seq) analyses. One normal HSC population (CD34⁺CD38⁻CD90⁺Lin⁻) and nine paired functionally defined LSC populations at diagnosis and relapse from six AML patients were shown in here. Mean $\pm$ SEM values are plotted. Wilcoxon matched-pairs signed rank test is used for comparing the diagnostic versus relapsed AML samples.

(A)

(B)

Figure 3.10. Knockdown of IL1RAP reduces the gene expression of TNF- α in primary AML cells or U937 cells.

Expression levels of TNF- α , a NF- κ B target gene, in primary AML cells and U937 cells with or without knockdown of IL1RAP. Total RNA was isolated from sorted GFP⁺ cells. Expression levels are normalized to universal human reference RNA (UHRR) and the average of reference genes *GAPDH* and *HPRT1*. Data of U937 cells in the lower panel were generated from five independent experiments. Technical triplicates were performed in each experiment. Mean $\pm$ SD values are plotted. The two-tailed Unpaired t-test is used for comparing the states described. **p < 0.01, ***p < 0.001, ****p < 0.0001.

Chapter 4

Discussion of Findings and Future Directions

4.1 Significance of Findings

The leukemia stem cell model is commonly summoned to explain why the majority of patients relapse following initial therapy for AML. Patients initially responsive to chemotherapy are far less likely to respond to similar therapy following relapse (Forman & Rowe, 2013). This argues that the malignant stem cell populations driving relapse undergo changes following the initial therapy. Elucidating the mechanisms behind this evolution could shed light on why current therapies fail patients. To address this issue, we obtained primary peripheral blood (PB) or bone marrow (BM) samples from patients treated for AML who were able to achieve a morphologic remission following standard induction therapy and subsequently relapsed. In Chapter 2, we characterized the frequencies of the LSC populations as well as their surface antigen phenotypes and gene signatures. We demonstrated a previously unreported increase in LSC frequency between diagnosis and relapse. While LSC frequency has been reported to vary considerably between individual patients (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2010), prior reports have not examined the impact of therapy and relapse on LSC frequency. By using limiting dilution (LDA) analyses, for the first time we identified a 9 to 90-fold increase in LSC frequency for individual patients. When we examined the expression of CD34 and CD38, we did not identify a consistent change in any population defined by these antigens that corresponded to the LSC frequency expansion identified by LDA. By assessing the LSC activity for the four populations defined by either CD34 and CD38 or CD32 and CD38, we demonstrated two findings; first, for the majority of samples, whether using CD34 and CD38 or CD32 and CD38, we demonstrated an expansion of

the LSC activity to populations of cells resembling a more differentiated/mature phenotype in relapse; second, the level of engraftment, which has been used as a surrogate for stem cell frequency, rose dramatically between diagnosis and relapse for individual populations, in the AML patients that we transplanted similar or more equivalent MNC dose at diagnosis. Previously, multiple studies in mice have demonstrated that targeting leukemogenic events to committed progenitors can activate a self-renewal program and confer LSC activity (Krivtsov et al., 2006; Y. Wang et al., 2010). Blast crisis CML evolves from chronic phase CML where the LSC has a surface antigen phenotype of an HSC. In a previous report, they demonstrated the leukemic stem cell activity in cells with a surface antigen phenotype resembling that of the GMPs in patients with blast crisis CML (Jamieson et al., 2004). Our data is the first to demonstrate the expansion of LSC activity to phenotypically distinct populations in AML patients following treatment and relapse.

Surface antigens were thought be a useful tool for tracking LSCs; however, previous reports and our findings in Chapter 2 have demonstrated heterogeneity of surface antigen expression profiles in functional AML LSC populations (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2008; Taussig et al., 2010). Neither CD34, C32, CD38 or any of the additional nine previously published LSC associated antigens tracked with the increase of LSC frequency identified using LDA for the twenty-five diagnostic/relapsed paired samples examined. We then compared the expression of the above LSC antigens on cell populations containing functional LSCs at diagnosis and relapse as well as the populations not possessing LSC activity at diagnosis that acquired

LSC activity at relapse. While our data demonstrated that several of these antigens distinguish between leukemic and normal stem cell populations, none of the antigens showed a significant increase in frequency between diagnosis and relapse. Additionally, we identified a significant increase in the expression of TIM-3 and CD45RA on populations of cells that did not demonstrate LSC activity prior to therapy and acquired LSC activity at relapse; however, this two-fold increase was far below the change in LSC activity observed by LDA. Taken together, our findings demonstrate that the frequencies of previously reported LSC antigens do not track with the change of LSC activity following drug treatment and disease progression.

To determine how LSC signaling is impacted following relapse, we employed a limited gene expression analysis on functionally defined LSC populations at diagnosis and relapse by using qPCR based TaqMan[®] Low Density Array (TLDA). Consistent with a distinct signature between normal and leukemic stem cell population, the HSC populations clustered together and segregated separately from the LSC populations. We also demonstrated that LSC populations from the same patient were more likely to segregate with each other as opposed to segregation based on disease state (diagnosis vs. relapse), suggesting an inter-patient heterogeneity between AML specimens. When assessing changes in the signature between diagnosis compared to relapse, the majority of the gene signature remained stable between diagnosis and relapse. In a closer look at the gene expression change within individual patients, we demonstrated a small subset of genes dysregulated following relapse, including the up-regulated genes *ATP1B1*, *SERPINE1*, *C16orf5*, *FLJ13197*, *MAFF*, *IL1RAP* and the down-regulated genes *BCL6*,

WASF3, *ITGB2*, *IL6ST*, *IRAK3*. Little is known about the functional role of most of the above genes in AML LSCs. Some have been reported to be associated with tumor malignancy. High expression of *ATP1B1* was able to discriminate invasive from low malignant potential epithelial ovarian tumors (Ouellet et al., 2005). The role of *SERPINE1* (as known as plasminogen activator inhibitor 1, PAI-1) in cancer is associated with enhancing tumor progression, invasion, metastasis and clinical adverse outcome (Binder & Mihaly, 2008; Duffy, McGowan, & Gallagher, 2008). Similarly, another plasminogen activator inhibitor, *SERPINB2* (PAI-2), has been shown to be important for leukemia growth and LSC development (Ashton et al., 2012). Collectively, these dysregulated LSC signatures represent genes with potential relevance to human AML LSC evolution during the clinical course and provide diverse targets for future research in developing new AML therapies.

Of the up-regulated genes in relapse, *IL1RAP* alone has been reported to be important in AML and CML. Intriguingly, we also observed that *IRAK3* is down-regulated in functionally defined LSC populations following relapse. *IRAK3* belongs to interleukin-1 receptor-associated kinase protein family and functions as a negative regulator of Toll/IL-R signaling (NCBI), implicating the downstream signaling of *IL1RAP* may be up-regulated as well. Thus, in Chapter 3, we further examined the functional role of *IL1RAP* in AML bulk and stem cell populations. IL-1 receptor accessory protein (*IL1RAP*) is a co-receptor of IL-1 receptor (*IL-1R*) and IL-33 receptor (*ST2*) and plays a key role in inflammatory responses (Dinarello, 2009). *IL1RAP* has recently been found to be overexpressed in chronic myelogenous leukemia (CML) (Jaras

et al., 2010), AML (e.g., -7/7q-, normal karyotype) and high-risk myelodysplastic syndrome (MDS) patients (Askmyr et al., 2013; Barreyro et al., 2012). The level of *IL1RAP* expression was shown to be associated with a poor overall survival in patients with AML with a normal karyotype ($P = 2.2 * 10^{-7}$ [log-rank]; hazard ratio [HR] = 2.59; 95% CI, 1.81-3.72) (Barreyro et al., 2012). Antibodies against IL1RAP were shown to kill CD34⁺CD38⁻ populations in CML (Jaras et al., 2010) and AML (Askmyr et al., 2013). We found that IL1RAP is expressed on the cell surface in the majority of AML patients' bulk, CD34⁺CD38⁻, and CD34⁺CD38⁺ cells compared to their normal counterparts. More interestingly, for the first time we demonstrated the expression of cell surface IL1RAP was much higher on functionally defined LSC populations than normal HSCs. Additionally, the IL1RAP expression was still observed in patients following drug treatment and disease progression. These findings taken together implicate a relevant role of IL1RAP in AML during clinical course.

IL1RAP's role in AML has not been addressed clearly. In a previous study, Jaras et al. employed retroviral transduction of cord blood CD34⁺ cells with P210 *BCR/ABL1* and demonstrated a strong up-regulation of *IL1RAP* compared to control-expressing cells (Jaras et al., 2010). Interestingly, a recent abstract reported that enforced expression of IL1RAP in cord blood CD34⁺ cells using retroviral system induced features of a myeloproliferative disorder in NSG mice (Hansen et al., 2013) (ASH 2013). Using a lentiviral system and several AML cell lines, Barreyro et al. found that knockdown of IL1RAP decreased clonogenic capacity and increased cell death *in vitro* (Barreyro et al., 2012). Given their findings, we performed a series of functional assays (e.g., cell growth,

apoptosis, cell proliferation, cell cycle, CFU and xenotransplantation) in order to determine the role of IL1RAP in primary AML and U937 cells. We demonstrated IL1RAP expression is important in the regulation of cell growth, survival and proliferation in bulk AML cells as well as the clonogenicity and engraftment potential in stem cells/progenitors in AML. A possible explanation of why stem cell/progenitor ability is affected after IL1RAP knockdown may be through the inhibition of cell survival or proliferation as we observed in total AML cells. It is also possible the loss of IL1RAP expression alters self-renewal capacity or homing; we are actively addressing these possibilities. Investigation of the downstream signaling mechanisms for IL1RAP in LSC populations is also underway and a genetic knock-in rescue experiment will be performed to exclude the possibility of shRNA off-target effects.

Given the heterogeneity within AML, we understand there are many factors that could affect these results. Increased expression of IL1RAP between diagnosis and relapse was identified in only three of the seven patients we examined in detail and nine of the twenty-five patients for the entire cohort. We selected patients with higher expression of IL1RAP for functional assays, so that the impact on AML progenitors with lower levels of IL1RAP needs to be assessed. Our sample size is too small to study the impact of patient specific mutation status and karyotype. The original papers demonstrated an association with normal cytogenetics and -7 but mutation data were not provided. Therefore, in the future, we need to broadly validate our findings with a larger cohort of primary AML specimens. Furthermore, using an IL1RAP knockout mouse model may facilitate to decipher the role of IL1RAP in AML *in vivo*.

Our findings and those of others bring to light the possible roles of IL1RAP in regulating AML pathogenesis and leukemia stem cell/progenitor function. The two established signaling mechanisms for IL1RAP are through NF- κ B and MAPK/c-Jun pathways; both pathways can induce the transcription of important genes to promote anti-apoptosis and cell survival effects. Our preliminary data identified that inhibition of NF- κ B/STAT3 pathways induced significant cell death in primary AML bulk cells in agreement with previous findings (Frelin et al., 2005; Guzman et al., 2001), and we found a previously non-reported increase in the activation of NF- κ B in functionally defined LSC populations using RNA-seq. Our findings highlight the possibility that AML LSC populations maintain cell survival through IL1RAP/NF- κ B signaling during clinical course. Interestingly, a recent report identified a positive feedback NF- κ B/TNF- α loop in promoting leukemia-initiating cells (LICs) capacity in murine leukemia models (Kagoya et al., 2014). Therefore, further functional analyses are needed to confirm our hypothesis.

Our findings identifying IL1RAP highlight the utility of genetic screens comparing diagnosis and relapse primary AML samples from the same patients coupled with functional validation of the LSC compartment. Using lentiviral shRNA system and xenotransplantation model, we can study the *in vitro* and *in vivo* functional relevance of interested gene in primary AML specimens at diagnosis or in relapse, which is much closer to what happens in patients. Given the heterogeneity inter- and intra- AML patients during clinical course (as shown in Chapter 2), a knockout IL1RAP mouse model would be useful to support the discovery in a homogeneous physiologic microenvironment. Future works will focus on deciphering the downstream mechanisms behind IL1RAP in

regulating AML LSCs. In addition, whether the dysregulated genes we identified in Chapter 2 or in our future global genome-wide analyses are important to AML LSC function (e.g, drug resistance, clonal evolution, cell survival, self-renewal capacity, homing, etc.) will be determined in our future studies and may provide new directions for AML therapy.

4.2 Future Directions

The findings in this thesis provide us many new thoughts and bring more exciting questions for our future directions. In Chapter 2, we characterized the evolution of LSCs following drug treatment and disease progression. There are some questions we have not addressed in this chapter.

1. What is the impact of drug treatment and disease progression on LSC frequency in populations defined by CD34 and CD38 or CD32 and CD38?

One important question that is not addressed in our study is the impact of chemotherapy and disease progression on the frequencies of phenotypically distinct LSC populations. While the LSC frequency in bulk cells increases 9 to 90-fold following relapse, it is unknown if this increase is shared equally by all LSC populations between diagnoses to relapse. The level of engraftment has been used as a surrogate for stem cell frequency. For the paired samples where similar cell doses were transplanted for an individual population, we found the frequency of leukemic engraftment in LSC populations were much higher at the time of relapse. This suggests that LSC frequency increases not only for the bulk population but for individual populations as well. Sarry et al. recently reported that the absolute distribution of LSCs does not correlate with the distribution of immunophenotype, but they did not study AML samples collected at different clinical courses (Sarrry et al., 2011). Thus, further works should include the quantitative evaluation of LSC frequencies using limiting dilution analyses to clarify the absolute LSC number in four populations from diagnosis and relapse samples.

2. How does the expansion of LSC activity occur following drug treatment and disease progression?

Our findings suggest the hierarchical organization was lost after standard chemotherapy and disease progression. We speculate that there are three possible explanations of how this occurs. First, these phenomena may result from the sequential acquisition of additional mutations in non-LSC populations during clinical course. Performing whole genome sequencing (WGS) or next-generation exome sequencing on functionally defined populations at diagnosis, remission and relapse will help address this question. Single cell genotyping would be more powerful. Similar studies have been done on blast cells in AML (Ding et al., 2012), secondary AML (Walter et al., 2012) and ALL (Mullighan et al., 2008). In addition to somatic changes in the DNA, epigenetic modifications have recently been shown to undergo significant change between diagnosis and relapse in patients with AML. Examining the epigenetic changes that occur in non-LSC to LSC transformation by performing ChIP-sequencing (ChIP-seq) on all four populations in paired pre-therapy and relapsed samples will also inform us on the underlying processes. A third potential mechanism is through chemotherapy selection of minor LSC clones that are below the detection sensitivity of current xenograft assay at diagnosis. Recent WGS data suggests that this is highly likely. To test this hypothesis, it will require a quantitative evaluation of pre-therapy and post-relapse LSC frequencies in all four populations with more sensitive mouse models.

3. Are there other ways to determine whether LSC associated immunophenotype can be used to track LSCs during clinical course?

In Chapter 2, we demonstrated the frequency of previously reported LSC antigens did not appear to track with LSC activity in the course of patients' disease. In this immunophenotyping assay, data analyses were performed in two-dimensional range gate using FlowJo software. Since AML is a very heterogeneous disease, the traditional gating strategy may not reveal the most relevant outputs. Thus, a new means to simultaneously measure multiple markers in individual cells may be helpful to address this question. Recent studies reported a novel technology, viSNE, which can map high-dimensional cytometry data onto two dimensions but maintain the high-dimensional structure of the data (Amir el et al., 2013). This tool is able to visualize the shape of the data generated from any multi-dimensional single-cell technology (e.g., mass cytometry). By using this new software to comprehensively analyze the expression of eleven LSC markers (CD34, CD38 and nine previously published LSC associated antigens) on paired diagnostic and relapsed samples, we may re-scrutinize the possibility to use these antigens to track LSCs during clinical course.

4. Functional validation of dysregulated genes and global approaches to characterize LSC specific signatures

In addition to the above questions, there are more investigations we are able to perform in the future. First, functional validation studies will be required to define the role of the up- or down-regulated genes identified in functionally defined LSC

populations from diagnosis to relapse. A functional role for these dysregulated genes in AML LSCs has not been investigated. Thus, similar approach of *in vitro* and *in vivo* functional assays as in Chapter 3 will need to be performed on primary AML and leukemia cell lines. Paired AML patient samples will also be used to determine the functional role of these dysregulated genes in LSC populations during clinical course. In addition, more global approaches will be helpful to examine the changes of molecular signatures of functionally defined LSC populations during evolution as well as the clonal expansion from non-LSC to LSC fraction between diagnosis and relapse. Currently we have performed RNA-seq analyses on functionally defined LSC and non-LSC populations from six pairs of AML patients at diagnosis and in relapse and more samples will be added to this analysis as they become available. Since our findings and others have demonstrated that LSCs are rare populations and the LSC frequency ranges various between patients, it is likely that the non-LSC cells within the surface antigen defined populations dilute the changes of LSC signature. It would be important to perform single cell sequencing to further investigate the changes of molecular signatures closer to what happens in LSCs.

In Chapter 3, we further demonstrated the functional role of IL1RAP in AML bulk and LSC populations and identify NF- κ B pathway may be a potential mechanism behind IL1RAP function in AML. Our findings create many interesting questions for future studies.

1. Is NF- κ B pathway the mechanism underlying IL1RAP in AML pathogenesis and LSC function?

IL1RAP is a co-receptor of IL-1 and IL-33 signaling, and both signaling mediates the downstream NF- κ B and MAPK pathways related to regulation of cell survival (Dinarello, 2009; Kakkar & Lee, 2008). Based on our preliminary data in Chapter 3, we hypothesize that IL1RAP regulate cell survival in AML bulk cells and LSCs through NF- κ B pathway. To test this hypothesis, there are some questions that need to be addressed. First, we have demonstrated knockdown of IL1RAP is able to induce significant cell death and impairs leukemic CFU and engraftment potential. For further analyses, we need to determine whether knockdown of IL-1 receptor (IL-1R) and IL-33 receptor (ST2) can achieve similar effects. Second, we found the inhibitors of NF- κ B/STAT3 pathways induced cell death in primary AML bulk cells while inhibitors of MAPK/c-Jun pathway did not. To validate these findings, we will confirm that these pathways are active in bulk and LSC populations using traditional western blotting or flow cytometry and whether they are effectively targeted by the drugs. Moreover, treated cells will be plated in methylcellulose-based matrix or transplanted into recipient mice to determine whether the inhibitors affect colony forming or engraftment ability. In order to examine whether the activation of NF- κ B pathway is reduced with IL1RAP knockdown, we will test the nuclear translocation of NF- κ B p65 using cell fractionation lysates from primary AML and U937 cells with or without IL1RAP knockdown. Additionally, by using ImageStream (amnis) or flow cytometry, we can further detect the Phospho-NF- κ B p65 in functionally defined LSC populations.

2. Are there other alternative downstream mechanisms of IL1RAP in regulating AML pathogenesis and LSC function?

Given that NF- κ B and MAPK/c-Jun pathways are two well-known downstream mechanisms of IL1RAP, it is possible that there is other non-conical pathway underlying IL1RAP in AML. To globally investigate potential mechanisms regulated by IL1RAP in AML, deep RNA-seq analyses will be performed on primary AML bulk cells and functionally defined LSC populations with or without IL1RAP knockdown. However, due to the limited materials, it could be difficult to perform above RNA-seq experiment on functionally defined LSC populations. An alternative approach is to perform *in vivo* small hairpin RNA (shRNA) screening to identify genes that can rescue the impairment of ILRAP knockdown in AML LSCs.

3. How can IL1RAP be used as a therapeutic target in the clinic?

A previous phase I study in patients with relapsed and refractory acute myeloid leukemia has shown that treating patients with the recombinant human interleukin-1 receptor (rhu IL-1R) was safe but did not have any antileukemic effect (Bernstein et al., 1999). The rhu IL-1R is a soluble truncated form of IL-1R and designed to compete ligand IL-1 with membrane-bound IL-1R in order to block IL-1 signaling. Although the rhu IL-1R treatment did not demonstrate efficacy, there are still many factors need to be considered. The results may be affected or limited by the small sample size ($n = 11$), patient status (relapsed and refractory AML), doses or patients' heterogeneity, etc. It is

also likely that IL-1 signaling was occurring in the intracellular space. Since we have demonstrated IL1RAP knockdown induced a significant impairment of cell survival, clonogenicity and engraftment potential in AML bulk or LSC populations, identifying compounds or antibodies to target IL1RAP should be strongly considered. Currently, a pharmaceutical company is developing therapeutic antibodies against IL1RAP and tests it on leukemia and melanoma (Cantargia). The mechanism is mainly through ADCC effect. To predict pharmacological compounds against IL1RAP associated function for antileukemia therapy, we could use Broad Institute's Connectivity Map (C-Map) database (Lamb, 2007; Lamb et al., 2006). Briefly, the C-Map is a database collecting thousands of genome-wide expression profiles derived from cultured human cells treated with small molecule agents. The software can match gene expression profiles derived from investigator with small molecule profiles in the C-Map to generate compounds that is able to induce most common gene expression changes. Similar drug discoveries were done in leukemia in previous reports (Ashton et al., 2012; Hassane et al., 2010). Thus, it will be of interest to use the RNA-seq profiles generated from primary AML cells with or without knockdown and perform c-Map analysis. Moreover, predicted compounds could further be used on testing whether it can reduce chemotherapy drug resistance of LSC populations *in vivo*.

4.3 Conclusions

In summary, the studies presented in this Thesis first characterize the evolution of AML LSC populations *in vivo* following drug treatment and disease progression. We demonstrated an increase in LSC activity and greatly increased phenotypic diversity of the LSC population from diagnosis to relapse, suggesting a loss of hierarchical organization following relapse. These findings demonstrate that the conventional chemotherapy regimens for AML patients may promote quantitative and qualitative expansion of the LSC compartment. Further, our data indicate the immunophenotype of surface LSC associated antigen is not predictive of function in relapse and suggest a major limitation to efforts targeting specific surface antigens in the relapse setting. In addition, we identified that IL1RAP is overexpressed in AML at diagnosis and in relapse compared to NBM and is critical to LSC properties. Loss of IL1RAP overexpression induced cell death and reduced clonogenicity and engraftment potential of primary AML or U937 cells, which implicates an important functional role of IL1RAP in AML pathogenesis and stem cell/progenitor populations. Thus, targeting IL1RAP is likely to improve our currently poor treatment options for patients who relapse. Taken together, our findings highlight the impact of current chemotherapy and disease progression on LSC evolution and suggest further therapeutic targets for drug discovery in AML treatment.

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