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Establishing a clinically relevant mouse model of human AML to test novel transmethylation inhibitors.

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ESTABLISHING A CLINICALLY RELEVANT MOUSE MODEL OF HUMAN AML TO TEST NOVEL TRANSMETHYLATION INHIBITORS

By

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B.S. in Biology, University of Louisville, 2008

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A Thesis Approved on

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ABSTRACT

ESTABLISHING A CLINICALLY RELEVENT MOUSE OF HUMAN AML TO TEST NOVEL TRANSMETHYLATION INHIBITORS

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Acute myeloid leukemia (AML) is a highly heterogeneous clonal disorder characterized by an accumulation of malignant immature myeloid progenitors in the bone marrow (BM) that hinder normal hematopoiesis. Patient AML exhibits a dramatic heterogeneity in terms of cytogenetics, disease morphology, and associated prognoses and/or chemotherapeutic sensitivity. Thus it becomes clearly evident that the investigation of novel therapeutics for AML will require model systems that are capable of recapitulating this stark heterogeneity in a patient specific manner. Furthermore, it is now understood that the surrounding bone marrow (BM) microenvironment and supporting cells play a critical role in leukemic progression as well as providing a chemotherapy protected sanctuary for residual disease. Therefore, the focus of this study was the establishment and development of a more clinically relevant mouse xenograft model of patient derived AML that not only recapitulates patient disease but also simulates the clinical standard of care induction therapy. The crux of our model system was the NRGS mouse, which were not only capable of reliable high rates of engraftment of established cell lines and patient derived AML cells, but also expresses three human myeloid cytokines (IL-3, GM-CSF, SF). Additionally these mice were able

to tolerate aggressive induction therapy at doses similar to those administered to patients, and therapy was efficacious in prolonging the survival of mice engrafted with patient AML. Such model systems that can simulate patient specific AML along with the standard of care therapy, will be essential for the successful investigation of novel, translational therapeutics.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

Figure Page

INTRODUCTION

Acute myeloid leukemia (AML) is a highly heterogeneous cancer characterized by an accumulation of malignant immature myeloid progenitors in the bone marrow (BM), peripheral blood, and rarely the central nervous system. These malignant cells proliferate rapidly and accumulate in the BM disrupting normal hematopoiesis. Recent studies have shown that these cells arise through the clonal expansion of myeloid committed hematopoietic stem cells (HSC) that have accumulated abnormal genetic alterations with age, resulting in a block in normal hematopoietic differentiation and aberrant proliferation. Thus AML exhibits a dramatic heterogeneity in terms of cytogenetics, disease morphology, patient prognosis and exhibits and an even more pronounced molecular diversity.

AML is the most predominant leukemia in older patients, and the dramatic increase in modern life expectancy is causing the incidence to skyrocket. The incidence of new cases of AML has risen by 2.2% every year for the last 15 years. There will be an estimated 21,000 new cases of AML diagnosed this year in the U.S. Luckily, modern chemotherapy allows approximately 60-70% of patients to achieve CR, but unfortunately the overall 5-year survival remains poor at only 26%, due largely to relapse.[1] The extreme heterogeneity of disease genetics and morphology presents a unique challenge for oncologists, as each case of AML is unique with its own associated prognosis and chemotherapeutic

sensitivity. Therefore novel, patient tailored therapies and/or treatment paradigms that are more efficacious, specific toatient cytogenetics, and better tolerated by elderly patients, must be developed in conjunction with standard of care therapy.[2]

As mentioned previously the cytogenetic heterogeneity of AML has long been recognized as the most important prognostic marker, as AMLs of varied etiologies have extremely different morphology and drug sensitivity. Accordingly, several systems of classification have been developed using differences in genetics, cellular morphology, and immune phenotype. During the 1970s AML was classified according to the French-American-British classification system which relies mainly on morphology and immunological phenotype, along with cytochemical criteria to define eight major subtypes (FAB M0 to M7).[3] The fundamental basis of this classification system was established by light microscopy examination of malignant cells as well as some limited cytogenetics characterizing recurrent genetic abnormalities. The numerical hierarchy of this classification system corresponds with both the degree of maturity (lowest being most stem) as well as the progenitor cell type from which the leukemia developed.

While groundbreaking in its novelty and recognition of morphology and some cytogenetics as key determining factors in overall prognosis, this system was found to be lacking in some instances. FAB classification worked well in instances where observed genetic lesions directly induced observed cellular morphology and cytochemical signaling. For example in acute promyelocytic

leukemia (APL) or acute myelomonocytic leukemia with abnormal eosinophils (M4Eo) the morphological characteristics directly predict the genetic abnormalities making these classifications very accurately.[4] However as research progressed it became more and more evident that morphology/cytochemistry did not always correlate with or predict observed genetic abnormalities, and differences in genetic abnormalities were frequently observed even in patients with the same proposed sub-type of AML. Additionally there are many cases in which there is absolutely no correlation between observed genetic defects and morphology, highlighting the fact that genetic profiling of AML provides a much more accurate system of classification and prognostic prediction. Thus, the FAB system was the first to recognize the immense morphological heterogeneity of AML and its value as a criteria for classification, but does not accurately convey the genetic diversity and associated clinical outcomes of the disease.

In 2001 the World Health Organization developed an alternate system of classification that is based on the central concept that the subgroups of AML are actually distinct diseases through correlation of genetic, morphologic, and clinical data. This new system of classification is significantly different from the FAB system as it utilizes a lower blast threshold for AML diagnosis and perhaps most significantly it defines cases of AML into unique subgroups based on biological, clinical, and genetic characteristics. The WHO classification system was updated in 2008 to its current format and defines seven major AML subtypes: 1.) AML with recurrent genetic abnormalities, 2.) AML with myelodysplatic related changes, 3.) therapy-related myeloid neoplasms, 4.) Myeloid sarcoma, 5.)

Myeloid proliferations related to Down Syndrome, 6.) Blastic plasmacytoid dendritic cell neoplasm, and 7.) AML otherwise not categorized (very similar to the FAB system with some additions).[4] Each major subgroup is divided into specific subcategories based on commonly observed recurrent genetic abnormalities, detailed karyotype analyses, and molecular abnormalities, thus providing a much better representation of the vastly different genetic landscape of AML even in morphologically similar cases. It becomes clearly evident that the WHO system of classification is far superior to the FAB system in terms of scope and descriptiveness, making it the chosen methodology for classification of AML in modern medicine.

Current chemotherapeutic treatment of AML is broken into two distinct phases, the first being induction therapy whose aim is to reduce the overall leukemic blast burden and induce remission. Unfortunately, intensive induction therapy has remained virtually unchanged since the 1970s and is effective in inducing CR in 60-70% of patients who are able to tolerate the therapy. Sadly, greater than 45% of patients achieving CR have a less than three year overall survival due to relapse and refractory disease. The standard of care induction therapy for young adults and healthy elderly patients is an intensive anthracycline and cytarabine regimen. Also commonly referred to as “7+3”, induction therapy utilizes a cytotoxic anthracycline like daunorubicin, idarubicin, or doxorubicin (60 or 90 mg/m^2) administered the first three days along with continuous intravenous infusion of cytarabine (Ara-C, $100mg/m^2$) for seven days. As previously stated the goal of induction therapy is to induce morphological remission which is

quantified as less than 5% blasts in the bone aspirate sample with marrow spicules and with a count of greater than 200 nucleated cells (must lack Auer rods and/or extramedullary disease). There must also be a platelet count greater than or equal to 100,000 cells/ml of blood and an absolute neutrophil count that is greater than 1000 cells/ul.[5-7]

Consolidation therapy, the second phase of current standard of care AML therapy, aims to prevent relapse and reduce the minimal residual disease (MRD) or leukemic blasts and leukemic founder cells protected from cytotoxic drug exposure in the BM microenvironment. These cells are the causative agents of refractory relapse, the major cause of patient mortality, and no current approved treatment regimens exist for relapsed AML. Oncologists have two options for consolidation therapy: 1.) Additional chemotherapy with intermediate dose cytarabine (1.5g/m^2) twice daily on days 1, 3, and 5 in 3-4 cycles or 2.) HSC transplantation (HSCT).[5] These strategies can be used at the clinician's discretion alone or in combination depending on the patient's subtype of leukemia, ability to tolerate chemotherapy, and availability of compatible donors. Chemotherapeutic consolidation therapy (3-4 cycles) alone has been shown to effectively improve survival and lengthen remission in patients less than 60 years of age, leaving HSC transplant as a last resort in the result of relapse. Comparisons of high dose (3 g/m^2) and intermediate dose (1.5 g/m^2) cytarabine show equal benefit for patients under 60 years of age and therefore the intermediate dose became the standard of care, while the high dose regimen

showed greater efficacy in patients with CBF AML [t(8:21); or inv(16)] or AML with mutated NPM1.[8,9]

In other prognostic groups allogenic stem cell transplantation remains the most effective long term therapy for AML after, with up to 60% of patients receiving transplant being cured. However numerous patients are deemed ineligible for transplant due to failure to achieve CR, co-morbidities, or lack of suitable donors. Initially age was thought to be the only determining factor in deciding whether or not to use HSCT, but new evidence conclusively shows that transplant success is dependent instead on active remission status and co-morbidities. Also improvements in conditioning regimens, supportive care, and haplo-identical/cord grafts have allowed a much larger segment of the patient population to be eligible for HSCT. Still the risk of relapse remains significantly higher in patients treated with HSCT alone and many patients are unable to receive transplant.[10,11]

A special case exists for only patients who are suspected of having the APL subtype of AML, which is treated with all trans retinoic acid even before genetic analyses are completed and disease presence confirmed. This therapy is clinically approved, even pre-diagnosis, and potently induces granulocytic differentiation in malignant APL blasts inducing apoptosis, quiescence, and increased cytotoxic drug sensitivity. Induction therapy is initiated in combination with ATRA upon confirmation of disease, and the combination of induced differentiation and cytotoxic chemotherapy has produced ground breaking results in multiple clinical trials worldwide. Rates of CR were 80-90% and rates of long

term survival conservatively exceed 75% changing APL's former grim prognosis into the most treatable form of AML. Considering AML is inherently different from other cancers in that AML blasts are essentially immature cells blocked in differentiation, novel therapies that force differentiation and maturation are highly attractive strategies for the treatment of AML as demonstrated by the success of ATRA in combination with induction therapy. Further study is required to develop new drugs that are capable of inducing differentiation in multiple or hopefully all types of AML, as demonstrated by the amazing improvement in survival and CR rates of APL treated with ATRA.[12]

Chromosomal irregularities (deletions, tandem repeats, translocations etc.) are observed in the majority of all primary AML cases (approximately 52%) and have long been recognized as causative events that cause and promote leukemic progression.[8] Each of these chromosomal abnormalities can be correlated with varied treatment outcomes, patient survival, and duration of remission making cytogenetic analysis of AML a critical factor in determining treatment options and how aggressively the leukemia is treated. For example, AMLs that have t(8;21)(q22;q22), inv(16)(p13.1q22), or t(15;17)(q22;q12) have a universally better prognosis with prolonged survival time as compared to other irregularities like alterations involving chromosomes 5 and 7 (complex karyotype AML) or 11q23 are associated with much shorter survival and diminished chemotherapeutic sensitivity.[13] Often times these genetic abnormalities result in the expression of characteristic abnormal fusion proteins (AML-ETO, PML-RARA, MLL fusions, DEK-CAN, etc.) many of which have been directly linked to

the observed block in differentiation and stem like phenotype of AML cells. Interestingly, approximately 40-50% of all AML cases are actually cytogenetically normal (CN-AML) using conventional analysis,[14] showing conclusively that AML associated genetic irregularities are not the only factor at play during the development of myeloid leukemia. Recent work is now starting to show that the presence or absence of specific mutations and/or altered regulation of gene expression can be used to further differentiate various types of AML (especially CN-AML) and possibly provide novel avenues of targeted therapy. This would allow treatment of AML in a patient specific manner, thereby greatly improving survival in patients harboring specific mutations or irregular patterns of gene expression regulation resulting molecular abnormalities.

Experts are now only starting to understand the effects of dysregulated patterns of gene expression and mutation status and how these effects mediate leukemogenisis.[4,15,16] This is of special importance in people diagnosed with CN-AML which appears normal according to karyotyping analysis, and lack the obvious genetic lesions found in other subtypes of AML. As such the prognostic value of cytogenetic analysis is limited in these patients, but with the advent of next-generation sequencing the genetic portrait of CN-AML has become much clearer. It is now understood that on average CN-AMLs have 13 mutations, five of which are recurrent “driver” mutations (directly causing leukemogenesis) and eight are recurrent random “passenger” mutations.[15] These mutations result in molecular abnormalities in the proteins produced, and these abnormalities are now being identified as key mediators of AML pathogenesis, as well as valuable

prognostic markers accurately predicting clinical outcome, and as novel druggable targets for new therapeutic approaches. Several mutations have come to the forefront as they have extremely high relative frequency in AML, and the most significant will be discussed here in brief.

Nucleophosmin 1 mutations (NPM1) mutations are by far the most recurrent molecular abnormality observed in AML and can be found in approximately 25-30% of patients.[17,18] Mutations in *NPM1* result in expression of an aberrant protein that shows abnormal cellular localization in the cytoplasm instead of the nucleus and this expression greatly promotes myeloid proliferation and leukemogenesis.[18,19] AMLs having these mutations generally present with a monocytic morphology with a generally favorable outcome in the absence of other mutations (namely FLT3 internal tandem deletions). This is perhaps the most clearly evident demonstration of the importance of deep-sequencing AML to examine potential molecular abnormalities as a way to guide therapy. AMLs with *NPM1* mutations have been conclusively shown to have a greater sensitivity to standard of care induction chemotherapeutics and as such the overall survival is greatly increased in both old and young patients as compared to other molecular abnormalities.[20] However *NPM1* mutations are also associated with several other recurrent abnormalities like DNA methyltransferase 3A (*DNMT3A*), *FLT3* internal duplications (*FLT3-ITD*), and isocitrate dehydrogenase mutations, most of which in conjunction with *NPM1* mutations actually worsen the prognosis.[21]

Fms-Like Tyrosine kinase (FLT3) was first identified in 1991 as a crucial regulator of normal hematopoiesis and plays highly important roles in mediating

cell survival and proliferation during hematopoiesis.[22,23] Internal tandem duplications (ITD) in region of the *FLT3* gene coding for the juxta-membrane domain or second tyrosine kinase domain of this tyrosine kinase have been found in approximately 20% of all AML cases and 30-45% of CN-AML cases.[24] These mutations create constitutively activate FLT3 promoting aberrant proliferation in leukemic blasts.[25] Thus patients with AML expressing *FLT3-ITDs* exhibit extreme leukocytosis and characteristic nuclear invagination, and these mutations are universally associated with poor prognosis, reduced survival and marked increase in risk of relapse. As expected the occurrence of these mutations in combination with other commonly recurring AML mutations (including those predicting better outcome) is correlated with diminished survival and increased rate of relapse. Interestingly, studies have also shown that non-juxta membrane domain mutations are associated with a worse prognosis as compared to mutations occurring in this region, again showing the clinical significance of mutations resulting in molecular abnormalities as a predictive biomarker for patient outcome and to guide therapy.[26]

Mutations of the *DNMT3* gene occur in a reasonable amount of AML cases (approximately 18-22% of all cases, 34% in CN-AML), the most common being missense mutations affecting arginine codon 822, while less common mutations in other codons have also been observed.[21] These mutations have now been found to actually be pre-leukemic and arise very early in the evolution of the disease, but also persist even in times of CR. This finding is significant in that these mutations are perhaps the most common mutations involving proteins

that regulate gene expression in AML.[27] DNMT3A is a *de novo* methylase that is capable of methylation of CpG dinucleotides in the promoter regions of target genes thereby mediating transcriptional silencing by blocking access of transcriptional machinery to these promoters. This form of regulation, which is independent of the genetic sequence, is known as epigenetic regulation and only very recently have methylation and other epigenetic mechanisms become recognized as relevant to leukemogenesis.[28] Therefore it is logical that these mutations occur very early in leukemic development, and may even play a causative role in AML, by silencing genes required for maintenance of normal myeloid differentiation and hematopoiesis. Finally, the value of *DNMT3A* mutations as prognostic indicators remains hotly debated with some smaller studies finding a lowered overall survival (OS) and disease free survival (DFS) in patients expressing these mutations, while a much larger cohort study found no correlation between *DNMT3A* mutation status and clinical outcome.[21,29]

These three mutations have the highest relative frequency of recurrence in AML patients, but it is important to recognize that they only represent a small fraction of the vast array of genetic and molecular abnormalities found in AML. It becomes clearly evident that AML has a heterogeneity that is one of the most extreme in all known cancers, and these differences result in drastically different treatment modalities and patient prognoses. As such the development of novel targeted therapeutics and treatment paradigms requires *in vivo* model systems that are capable of faithfully recapitulating the extreme genetic and morphologic variability found in patient AMLs. Furthermore such systems must also be able to

replicate standard of care induction therapy, not only as a more relevant control (as compared to vehicle only), but also as a more clinically applicable way to test experimental therapeutics and their translational efficacy.

Animal models have been consistently shown to better predict the success of experimental therapeutics in clinical trials, and this holds especially true for the investigation of new drugs for AML, as a complex and highly dynamic interaction exists between heterogeneous populations of leukemic cells and the surrounding BM microenvironment.[30] Current evidence supports the conclusion that specific niches within the BM microenvironment actually provide a protected sanctuary for specific subpopulations of leukemic cells capable of self-renewal and clonogenic proliferation, dubbed leukemic stem cells (LSCs). Seminal studies using early xenograft models were able to establish that only the most primitive fraction (Lin^- CD34+) of patient AML cells, and not the more mature blast population, were capable of transferring disease to primary and secondary NOD/SCID recipient mice.[31,32] It becomes evident that a hierarchy similar to normal hematopoiesis exists in AML, with a small population of self-renewing LSCs giving rise to a large population of mature, clonally expanded blasts. LSCs are able to evade normal induction chemotherapy, due to a low rate of proliferation and protection conferred by the BM microenvironment, and are therefore thought to be the cause of the high rate of relapse and mortality associated with AML. Perhaps more alarming is the mounting data that these LSCs can actively induce changes in the BM microenvironment not only at the structural level but in cytokine production and genetic/epigenetic abnormalities within supporting BM stromal

cells.[33,34] Co-culture experiments have shown that HSCs cultured on BM stroma collected from AML patients have a pronounced impairment in both self-renewal and survival capacity, while LSCs lose neither attribute on AML or normal stroma.

Thus LSCs induce changes to the BM microenvironment and supporting cells which favor LSC survival and maintenance while simultaneously compromising its ability to support and maintain normal HSC survival and differentiation. These interactions add another layer of complexity, additional to the dramatic heterogeneity of AML, which again must be accounted for in model systems that will ultimately be useful in the development of successful therapeutic paradigms that will truly translate to clinical care. Again *in vivo* animal studies remain the only reliable way to examine the highly complex interplay between leukemic populations (LSC and blast populations) and the protected BM niche, and by extension the pharmacological efficacy of novel therapeutics. Finally, *in vivo* methodologies have been shown to have significant advantages in success rates of experimental drugs as compared to *in vitro* techniques which often show success in the lab, but due to the lack of an accurate organ specific extracellular environment, cross talk between hormone or different cell types, differential metabolism, and site specific delivery often fail to show efficacy in clinical trials. This remains especially true for AML as effective novel therapeutics must be able to target the largely quiescent and protected LSC population residing in the bone marrow, or alternatively target the bone marrow

microenvironment to make it unfavorable for leukemic growth or promote normal hematopoiesis.

The development of the first immune deficient strains of mice in the 1960s overcame a major technical hurdle required to create such more clinically relevant xenograft mouse models which allow for the engraftment of human cells. Attempts are now even being made to replicate human cytokine signaling through transgenic mice that expressing human cytokines, thus starting to model the exquisitely complex role of cytokine signaling in leukemic and normal hematopoiesis. These “humanized” models, in which human cells can engraft and function have become revolutionary in the study of both normal and malignant human hematopoiesis, and will play a crucial role in the development of new therapeutics and treatment paradigms.

Highly immune deficient mice have become indispensable to the study of both normal and malignant human hematopoiesis, as these mice do not reject human xenografts and support the differentiation and continued growth of human cells. Discovered in 1962, nude or athymic mice were the first immune compromised mouse strain capable of accepting human cells and tissues and soon after severe combined immune deficiency (SCID) mice.[35] SCID mice completely lack the ability to mount and sustain effective adaptive immune responses, due to an absence of functioning T and B lymphocytes, caused by a recessive mutation resulting in deficient activity of the enzyme; protein kinase, DNA activated, catalytic polypeptide (Prkdc). This enzyme is crucial in DNA damage repair as well as required for V(D)J recombination which explains how

this mutation disrupts the maturation and function of T and B lymphocytes. SCID mice were found to be far superior to nude mice in terms of engraftment of human tissues, and further refinement came with the development of non-obese diabetic (NOD)/SCID mice through the introduction of the *Prdkc*^{scid} gene into an inbreeding NOD strain.[36,37] These mice showed even greater rates of human cell engraftment as compared to SCID mice. Similarly, mice with that have a defective recombination activated gene (RAG, can be either Rag1/2^{null}) also display a SCID phenotype and were crossed with NOD mice resulting in NOD/Rag1/2^{null} mice also capable of reliable human tissue growth and engraftment.[38,39] Numerous iterations of transgenic (NOD)/SCID mice capable of expressing various human cytokines were developed in an attempt to further improve rates of engraftment and promote true multilineage hematopoiesis, growth, and differentiation of human hematopoetic cells.[40]

The early 2000s saw the advent of a series of immune deficient mice appropriate for the creation of truly “humanized” mouse models that showed not only high rates of engraftment, but also well-differentiated multilineage expansion and engraftment of human leukemia or HSCs. These mice were developed through the introduction of a mutant *IL2ry* gene into (NOD)/SCID mice through respective backcross mating, resulting in the generation of the two most widely used strains of immune deficient mice, NOG and NSG mice, which are (NOD)/SCID IL-2ry^{null} and differ only in the location of the SCID mutation.[41,42] Inactivation of IL-2ry, which is shared by several cytokines (IL-2, IL-4, IL-7, IL-15 and IL-21) crucial to T- and B-cell growth, allowed for much better multilineage

immune suppression and overcame the inherent “leakiness” observed in NOD/SCID mice, which eventually develop T- and B-cells with age.[43] This mutation was also introduced into and (NOD)/Rag1/2^{null} mice to generate NRG [(NOD)/Rag1/2^{null} IL-2ry^{null})] mice, whose immune impairment is dependent on hematopoietic specific RAG deficiency, thus leaving DNA repair intact.

Functionally these mice were a revolutionary leap in xenograft mouse models of human leukemia and show multiple immune deficiencies well beyond lymphocytic impairment. These mice exhibit defects in T, B, and natural killer (NK) cells as well as markedly reduced dendritic cell and macrophage function.[41] Extremely enhanced engraftment rates of human cell lines, HSCs, and even primary patient samples have been observed, as well as well-defined differentiation and maturation of multiple distinct hematopoietic lineages upon human HSC xenograft as compared to parent strains. Of particular importance was the finding that these mice support the differentiation and proliferation of human T-cells, specifically CD4 and CD8 single positive cells, which do not develop in NOD/SCID mice. Indeed these strains, particularly the NSG mouse, have become the workhorses for modeling and studying human leukemogenesis and hematopoiesis in an *in vivo* system that begins to attempt to mimic physiological conditions experienced by leukemic cells in the human body. Such *in vivo* systems that are capable of faithfully replicating physiological conditions, and support the proliferation of human or patient derived leukemia, are critical in the search for novel therapeutics that have a high potential of advancing to clinical trials and/or becoming approved therapies.

While these mice provided a revolutionary leap in the study of both normal and leukemic human hematopoiesis, they fail to model several important conditions experienced by human AML patients. The most glaring and obvious disadvantage is the lack of a fully functional immune system, especially the lymphocytic compartment which is severely impaired to allow for the engraftment and proliferation of human cells. Attempts are now being made to develop mice with “humanized” immune systems, and essentially rely on transplantation of normal human CD34+ HSCs into competent immune compromised mice. Several groups have been able to show well characterized differentiation along multiple well defined hematopoietic lineages, and most importantly the presence of human T and B lymphocytes. Unfortunately the utility of these “humanized” mouse systems remain insufficient as the resulting human cells are often times not fully functional, and fail to produce immunomodulatory molecules like cytokines and IgG upon antigen presentation.[44] Mouse models of human leukemia that can eventually incorporate a functional human immune system will be required for truly translational investigation of novel therapeutics, especially immune based therapies.

Second, and perhaps as important, is the lack of hematopoietic cytokine signaling as most mouse cytokines do not cross react with human cells. This is a major shortcoming as cytokine production and signaling is known to be required for both normal human hematopoiesis and plays a role in leukemic progression and clinical outcome. Additionally, this signaling is required for reliable homing, engraftment, and proliferation of both normal human HSCs, and xenograft patient

samples which are notoriously difficult to engraft.[45] To overcome this problem several groups have developed a series of NOG and NSG mice that express transgenes for a variety of human cytokines specifically geared to the desired area of research (e.g. hIL-2, hIL-3, hIL-6, hGM-CSF, etc.).[41,46,47] This development further improved these models by allowing for the engraftment, differentiation, and development of patient derived leukemia or normal human HSC in a BM microenvironment, as well as limited human cytokine signaling.

Despite these advances NOG and NSG mice have major flaw that is often overlooked, and is a result of a mutation in the *Prdkc* gene used to generate the SCID phenotype. This gene and its enzyme product Prdck are required for not only V(D)J recombination of lymphocytes but also DNA damage repair. As such these mice are highly intolerant to chemotherapeutics, especially DNA damaging agents, as they are unable to repair any of the damage caused by the drugs. This poses a significant challenge as the standard of care induction therapy for AML uses doxorubicin (or other anthracyclines like daunorubicin or idarubicin) and cytarabine, both DNA damaging compounds. Doxorubicin intercalates DNA thereby preventing macromolecular biosynthesis and through stabilization of topoisomerase II promotes breaks in the DNA. Cytarabine or cytosine arabinoside interferes with DNA synthesis due to rapid conversion to a toxic and DNA damaging intermediate, when the cell is in the S phase and replicating DNA. Thus mice harboring SCID immune deficiencies are extremely intolerant to treatment with these drugs, even at subtoxic doses in other mouse strains, a critical flaw. Truly translational models must be able to closely mimic the patient

experience, as well as model the standard of care therapy not only as a more relevant control, but also to investigate innovative and/or combinatorial therapeutics. Taken together a more clinically and translationally relevant mouse model of AML will require not only mice capable of receiving and supporting human patient xenografts, but also express human myeloid cytokines and tolerate aggressive chemotherapeutic treatment similar to clinical induction therapy.

Recently, Mulloy et. Al. developed a new strain of mice by crossing NRG mice with NSGS mice which are essentially NSG mice that harbor the SGM3 (3GS) triple coinjected transgenes that express human interleukin 3 (IL-3), granulocyte/macrophage colony stimulating factor (GM-CSF), and steel factor (SF). Upon genotyping of the offspring, selective breeding together or with NRG mice allowed for the removal of the SCID mutation resulting in NRGS mice which are essentially NRG mice that also express the SGM3 transgene. Thus these mice have no mature T cells, B cells, or NK cells due to the hematopoetic specific Rag mutation (as opposed to a SCID mutation) and express three human cytokines important for both engraftment and support of human myeloid cells. Since these mice do not depend on a SCID mutation to mediate immune suppression they have functional DNA repair and should be able to tolerate clinically comparable doses of chemotherapeutics and previous studies have shown that these mice maintain the radioresistance that is a hallmark of the NRG strain. This strain of mice is truly cutting edge in terms of modeling human hematopoiesis, both normal and malignant, as not only can these mice tolerate

aggressive induction chemotherapy and dose escalation, but also express three human cytokines necessary for the engraftment and support of human myeloid cells.

HYPOTHESIS

We hypothesize that we can develop a more clinically relevant xenograft mouse model of human AML for the investigation of novel therapeutics and treatment paradigms. This is essential, especially for AML which is a uniquely heterogeneous cancer that presents with a variety of morphologies and express a diverse array of genetic and molecular irregularities, each with their own associated treatment sensitivities and clinical outcomes. Our model is hinged on NRGS mice which will not only allow for high rates of human AML cell engraftment, but will also attempt to recapitulate some human myeloid cytokine signaling. Finally, the model must be resilient enough to tolerate aggressive induction chemotherapy at doses similar to those experienced by AML patients, and treatment with induction chemotherapeutics must provide a significant survival outcome in mice engrafted with human AML. Establishment of such a model will be crucial to the discovery of novel compounds, whose efficacy against patient specific AML can be tested against and in combination with standard of care induction therapy. Compounds identified in this model system are expected to have considerably improved success rates in advancement to clinical trials and hopefully improve patient treatment outcomes and remission rates.

MATERIALS AND METHODS

ANIMAL STUDIES

NRGS mice were obtained from Jackson laboratories and bred and maintained at the University of Louisville Rodent Research Facility (RRF) on a 12-hour light/12-hour dark cycle and provided food and water ad libitum. Animals were maintained under standard conditions and all animal procedures were approved by the Institutional Animal Care and Use Committee. In all studies the appropriate number of human AML cells from established lines, patient derived cells, or MYC/BCLXL induced murine leukemia were resuspended in PBS and tail vein injected at a final volume of 200 µl. Mice receiving “5+3” therapy were weighed daily and given a dose of 75 mg/kg of cytarabine for five days and 3 mg/kg doxorubicin for three days by bolus tail vein injection, with each injection being balanced with PBS such that each mouse receives a total volume of 200 µl. Mice in the vehicle groups received injections of 200 µl plain PBS for all five days. Upon development of leukemia or termination of the experiment mice were sacrificed and FACS analysis conducted on the spleen, thymus, and BM

REAGENTS AND ANTIBODIES

Clinical formulations of both doxorubicin and cytarabine were obtained from the James Graham Brown Cancer Center as single dose self-sealing vials containing 20 mg/10 ml or 2g/20ml respectively, manufactured by APP a division of Fresenius Kabi USA LLC (Lake Zurich, IL).

CELL CULTURE

Cells were maintained in culture at 37° C under 5% CO_2 in RPMI 1460 growth media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

PATIENT SAMPLES

Patient derived AML cells obtained from the spleen of an NSG mouse engrafted with the primary patient leukemia, were a generous gift from our collaborator Dr. James Mulloy, at Cincinnati Children's Hospital.

FACS ANALYSIS

Both the spleen and BM were analyzed by FACS. In brief, single cells were isolated from tissues; red blood cells were lysed and blocked for 10 mins at room temperature with Fc Block (#553142 BD Biosciences, Miami, FL, USA). The samples were then stained with anti-human CD45 antibody conjugated to the Alexa 700 fluorophore, from BioLegend (San Diego, CA) for 20 mins at 4° C and then analyzed on a Becton Dickinson FACScan with FlowJo.

RESULTS

Engraftment of multiple human AML cell lines in NRGS mice. First we wanted to examine the engraftment and subsequent disease progression of NRGS mice tail-vein injected with 1 x 10^5 human AML cells. Differential rates of engraftment based on leukemic subtype and classification were examined by injecting either OCI-AML3 cells or KG1 cells. Each of these cell lines represent dramatically different subtypes of AML and therefore the utility of this model for multiple cytogenetically distinct subtypes of AML encountered in the clinical setting. Three NRGS mice were injected with 1 x 10^5 of each cell line and monitored until humane endpoints were achieved. We found that both cell lines showed high rates of consistent engraftment (n=3) as quantified by the amount of detectable human CD45 in the spleen and BM by FACS. Mice injected with OCI-AML3 cells survived till approximately 30 days till human endpoints were reached, while mice injected with KG1 cells survived only 23-25 days. This data corresponds to these cell lines in vitro growth rate as well as their subtype associated aggressiveness. These data indicated that NRGS mice are capable of high rates of engraftment and fairly rapid disease progression when injected with established human cell lines, allowing for the establishment of model systems that are capable of investigating novel therapeutics against various human AML subtypes in an *in vivo* environment with the influence of human myeloid cytokines.

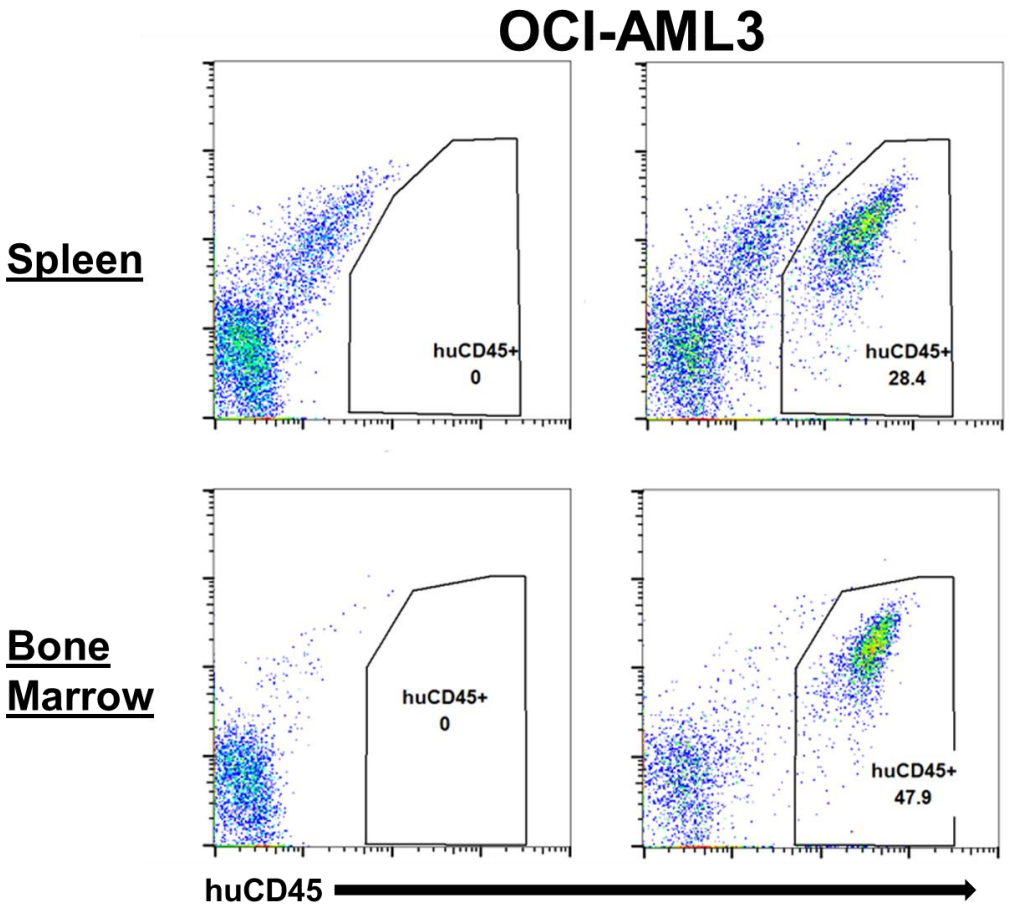

Figure 1. Engraftment of 1 x 10^5 OCI-AML3 cells in NRGS mice. NRGS mice (n=3) were injected with 1 x 10^5 OCI-AML3 cells and maintained under standard conditions until humane endpoints were reached as characterized by distress, breathing difficulty, hind limb paralysis, etc. Mice had to be sacrificed from 30-32 days after injection of cells and FACS analysis of the BM and spleen showed a large percentage of human cells (representative mouse shown). Approximately 30% of the cells in the spleen (A.) were human OCI-AML3 cells and surprisingly almost 50% of the BM. (B.) was comprised of human cells. The left column represents unstained cells, to ensure that results were irrespective of inherent cellular auto-florescence.

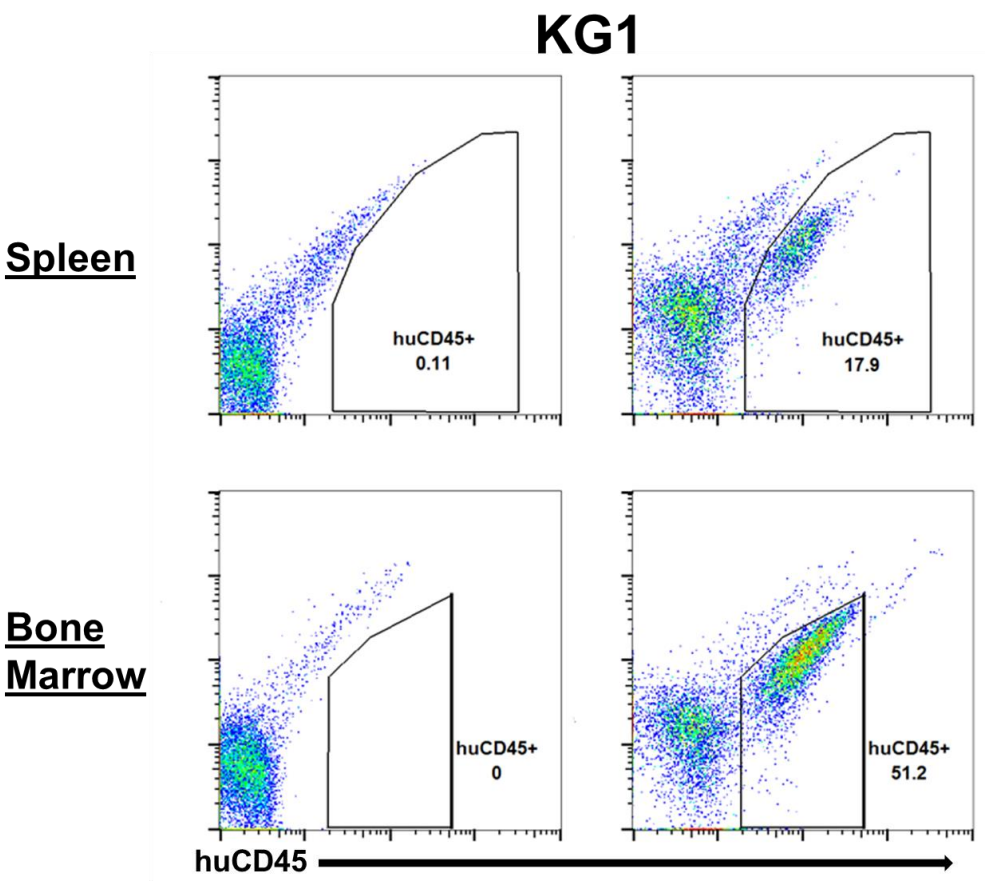

Figure 2. Engraftment of 1 x 10^5 KG1 cells in NRGS mice. NRGS mice (n=3) were injected with 1 x 10^5 KG1 cells and maintained under standard conditions until humane endpoints were reached as characterized by distress, breathing difficulty, hind limb paralysis, etc. Mice had to be sacrificed after 23-25 days after injection of cells and FACS analysis of the BM and spleen showed a large percentage of human cells (representative mouse shown). Only 18% of the cells in the spleen were human KG1 cells and again almost 50% of the BM was comprised of human cells. Again the left hand column indicates analysis of unstained cells to account for any possible inherent auto fluorescence of the cells.

Engraftment of patient derived AML cells in NRGS mice. Once we were able to establish that high rates of engraftment and predictable disease progression could be achieved in our model using established human cells lines, we wanted to progress to the engraftment of notoriously difficult human patient derived AML cells. Previous studies have demonstrated that similar immune deficient mouse models of human AML can accurately predict patient chemotherapeutic response, and faithfully maintain the unique genetic and phenotypic identity of engrafted patient AML. We also wanted to confirm similar findings in our model, these findings being a prerequisite to a truly physiologically, translationally, and clinically relevant mouse model of AML. Therefore, the cells were examined by FACS analysis for human myeloid markers both pre-injection and post sacrifice. Thus, NRGS mice (n=5) were tail-vein injected with 1.25×10^5 patient derived AML cells (as determined by FACS analysis for human CD45) and maintained under standard conditions until visible signs of leukemia were evident and humane endpoints achieved. All mice had to be terminated at 41 days after injection of cells, and most mice showed overt signs of leukemia, like hind limb paralysis. FACS analysis of both the BM and the spleen showed the presence of a large percentage of human cells with the same myeloid antigen expression as compared to cells pre-injection, indicating that engraftment of the patient sample was successful. Interestingly both CD14 and CD33 are virtually undetectable upon defrost, but are heavily expressed after transplantation into the *in vivo* environment. These data indicate that NRGS mice are valuable tools that allow for the robust engraftment of both human cell lines, and more importantly primary patient derived AML cells.

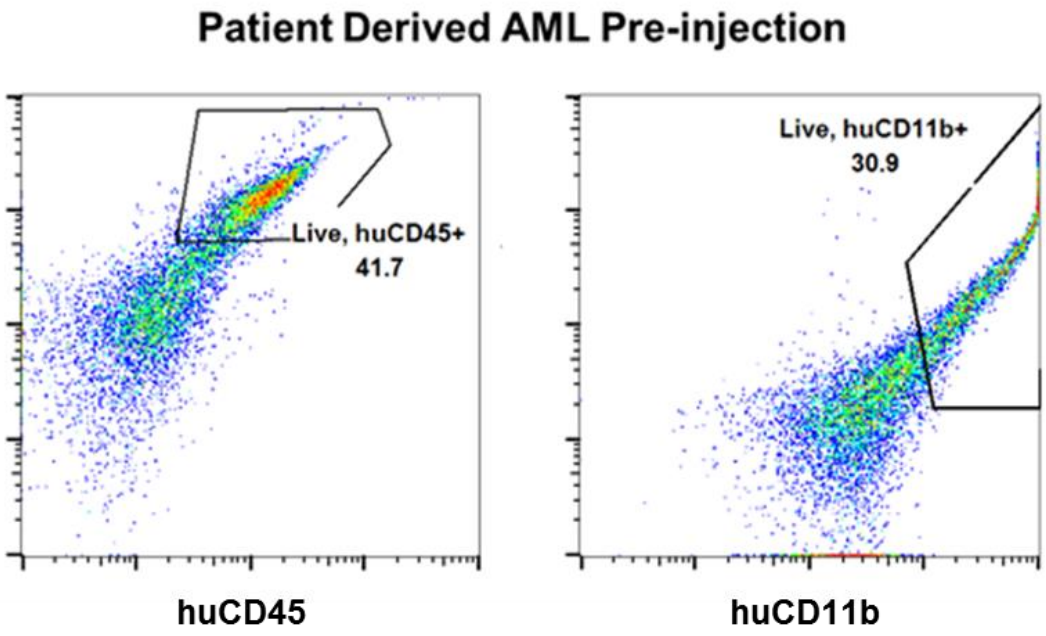

Figure 3. FACS analysis of human myeloid cell surface markers upon defrost and before tail vein injection into NRGS recipients. After thawing the cells FACS analysis was conducted using antibodies against human CD45, CD33, CD11b, and CD14. Since these cells were acquired from the spleens of NSG mice transplanted with the primary patient leukemia approximately 41.7% of the cells were of human origin as characterized by human CD45 expression. The cells were positive only for huCD45 and huCD11b and showed no expression of huCD33, a common myeloid cell surface antigen, or huCD14 an antigen expressed by a variety of mature hematopoietic lineages.

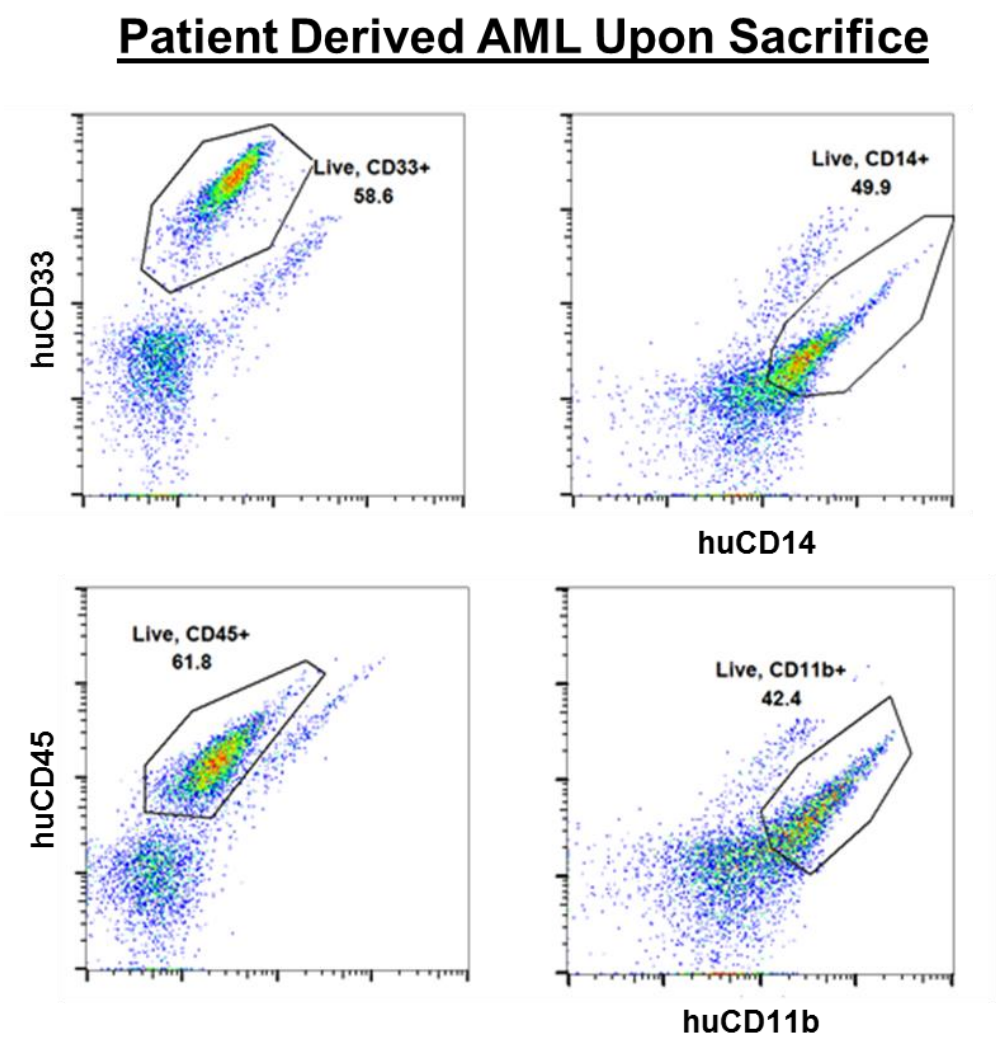

Figure 4. FACS analysis of human myeloid cell surface markers after injection and sacrifice of NRGS mice injected with 1.25 x 10^5 patient derived AML cells. After humane endpoints were achieved at 41 days, the mice were sacrificed and their hematopoetic organs were examined by FACS analysis to determine the percentage of human cells as well as examine the maintenance of cell surface marker expression. As expected there were a high percentage of human cells as identified by CD45 expression, and these cells maintained their human myeloid cell surface expression as compared to the patient cells upon defrost, with the exception of CD33 and CD14 which were detectable.

Treatment of leukemia in mice with a clinically similar dosing regimen of induction chemotherapeutics. As discussed in detail previously the current standard of care therapy for AML centers around administration of an anthracycline (doxorubicin, daunorubicin, idarubicn etc.) for three days combined with continuous infusion of cytarabine (Ara-C) for seven days and therefore often called “7+3”. Based on published studies from a collaborator, Dr. James Mulloy, we utilized his “5+3” regimen for mice which has been scaled down taking into account both body mass as well as differences in pharmacokinetics, but done using a different stain of mice. Therefore it was of crucial importance for us to investigate the efficacy of our treatment regimen in mice harboring leukemia, to be used as both a more relevant control and for investigation of combinatorial therapies. Thus, NRGS mice transplanted with patient AML cells were allowed to incubate for a week at which time they began daily tail vein injections of 75 mg/kg cytarabine for five days, and doxorubicin at 3 mg/kg for the first three days. Recognizing a major criticism of these mouse models, including the lack of a functional immune compartment and the feasibility issues associated with the acquisition of primary patient samples, we also decided to test the efficacy of this same chemotherapeutic regimen on wild-type FVB mice engrafted with our own in house transplantable murine myeloid leukemia. This leukemia was generated by retroviral induced overexpression of Myc and BCLXL in normal BM hematopetic progenitors as previously described. This would provide an easier and more high-throughput methodology for screening novel therapeutics against and in combination with standard of care therapies, along with the influence of a fully functional immune compartment. Additionally, Myc is

recognized as one of the most potent oncogenes and BCLXL an extremely potent proliferative signal making this myeloid leukemia extremely aggressive. Therapies showing efficacy in this model will therefore have a high likelihood of success in clinical trials against patient leukemias which are generally much less aggressive. Here we show for the first time that “5+3” therapy with doxorubicin and cytarabine results in a significant enhancement of survival in both NRGS mice engrafted with patient derived AML cells and in FVB mice receiving our MYC/BCLxL induced murine myeloid leukemia. The survival benefit was much more pronounced in the NRGS mice with patient derived leukemia resulting in an average increase in survival of 12 days as compared to the vehicle treated mice. FVB mice engrafted with MYC/BCLxL induced myeloid leukemia show a diminished but still significant response to “5+3” induction therapy, with survival being prolonged by an average of 7 days. Both these results indicate that NRGS mice are capable of handling aggressive chemotherapeutic treatment with standard of care therapy in a manner similar to the clinical experience of patients. Perhaps more importantly these data indicate that our treatment regimen is actually capable of treating both mice engrafted with patient derived leukemia and murine leukemia, a crucial finding, especially if this regimen is used as a control for efficacy and for studies of combinatorial therapy.

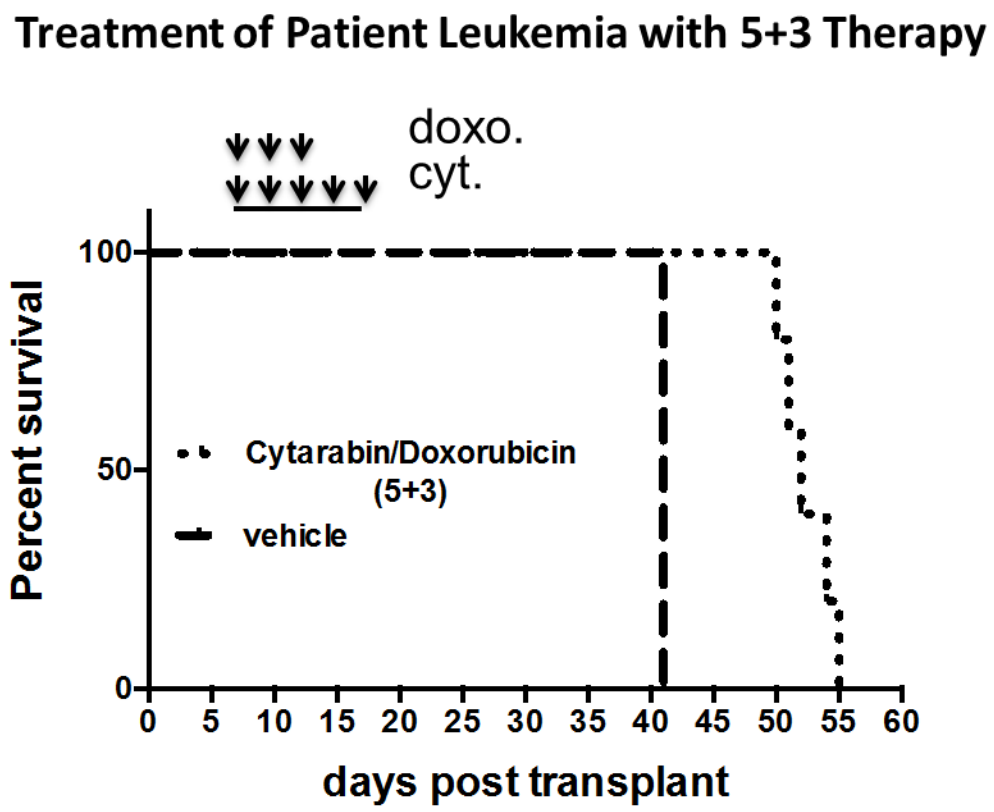

Figure 5. Treatment of NRGS mice engrafted with patient derived AML using "5+3" induction therapy. NRGS mice (n=10) were tail vein injected with 1.25 x 10^5 patient derived AML cells obtained from the spleen of an NSG mouse engrafted with the primary patient leukemia and maintained under standard conditions for seven days. Next one group (n=5) was given "5+3" induction therapy composed of 3 mg/kg doxorubicin for the first three days and 75 mg/kg cytarabine all five days, while the vehicle group received sham injections of the vehicle PBS. Survival was significantly prolonged by an average of 12 days indicating that our clinically modeled regimen is effective in the treatment of patient derived leukemia, a crux in the development of a more clinically relevant AML mouse model that better recapitulates patient experience, and also as a control during the investigation of novel therapeutics.

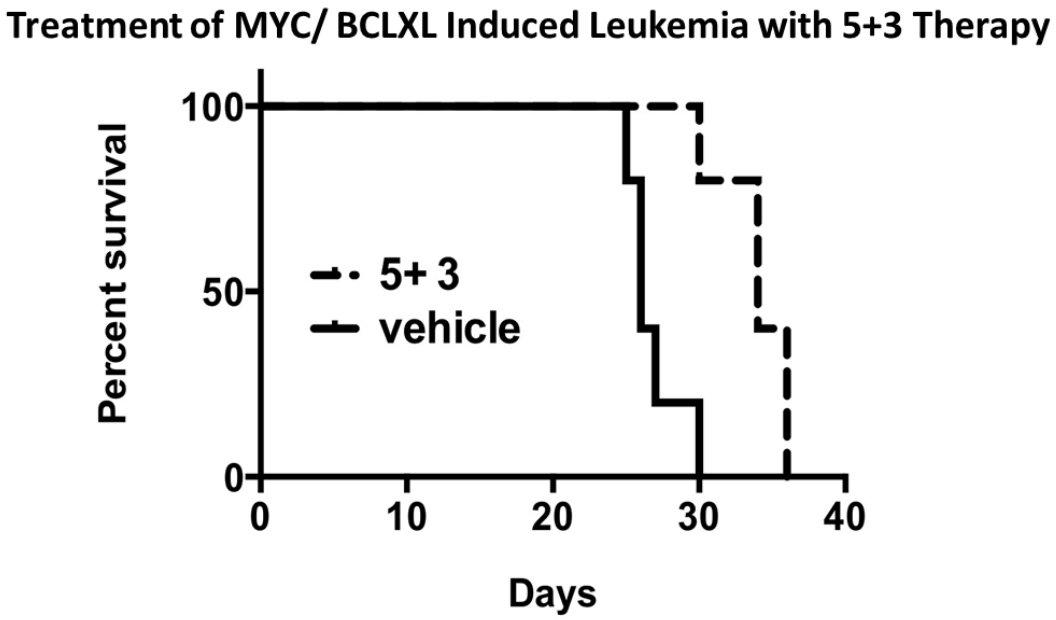

Figure 6. Treatment of MYC/BCLXL induced murine myeloid leukemia in wild type FVB recipients with functional immune systems. FVB mice (n=10) were tail-vein injected with 5 x 10^5 spleenocytes taken from an FVB mouse after development of MYC/BCLXL induced leukemia and sacrifice, and maintained under standard conditions for seven days. One group of mice (n=5) received "5+3" induction therapy with 75 mg/kg cytarabine administered all five days and 3 mg/kg doxorubicin for the first three days, while the vehicle group (n=5) received sham injections of PBS. Chemotherapeutic treatment significantly increased survival by an average of 7 days, indicating that our dosing regimen is also effective against an extremely aggressive murine myeloid leukemia, albeit with decreased efficacy as compared to treatment of patient leukemia. Most importantly this model has a fully functional immune system allowing for more physiologically accurate investigation of therapeutics, especially immune therapies.

Humanization of NRGS mice. The absolute pinnacle in the development of xenograft mouse models of patient AML would be the influence of a functional human immune system in addition to patient specific leukemia. Therefore we attempted to engraft sub-lethally irradiated NRGS mice (n=3) with human CD34+ multipotent HSCs obtained from umbilical cord blood, in the hope that we could observed multi-lineage expansion of human cells in the mouse microenvironment. Approximately 100 days after injection these mice were sacrificed and their spleens and BM analyzed by FACS analysis for the presence of several linage restricted human cell surface markers. Two of the three mice in this arm of the study showed the presence of human cells in the bone marrow, while all three mice showed no detectable human cells in the spleen. The mature human myeloid marker CD33 could be detected at low levels in the BM (3.54%, 9.34%), and the human B-lymphocyte antigen CD19 could be detected at higher levels (8.67%, 27.6%). Mouse #2 even stained positive for the human T-cell receptor CD3 at a detectable level (0.6%). These results demonstrate that NRGS mice are capable of being humanized upon sub-lethal irradiation and tail-vein injection of human CD34+ multipotent HSCs. The eventual goal of these studies is to incorporate a functional human immune system into our more clinically relevant patient xenograft AML mouse model, and these data demonstrate feasibility.

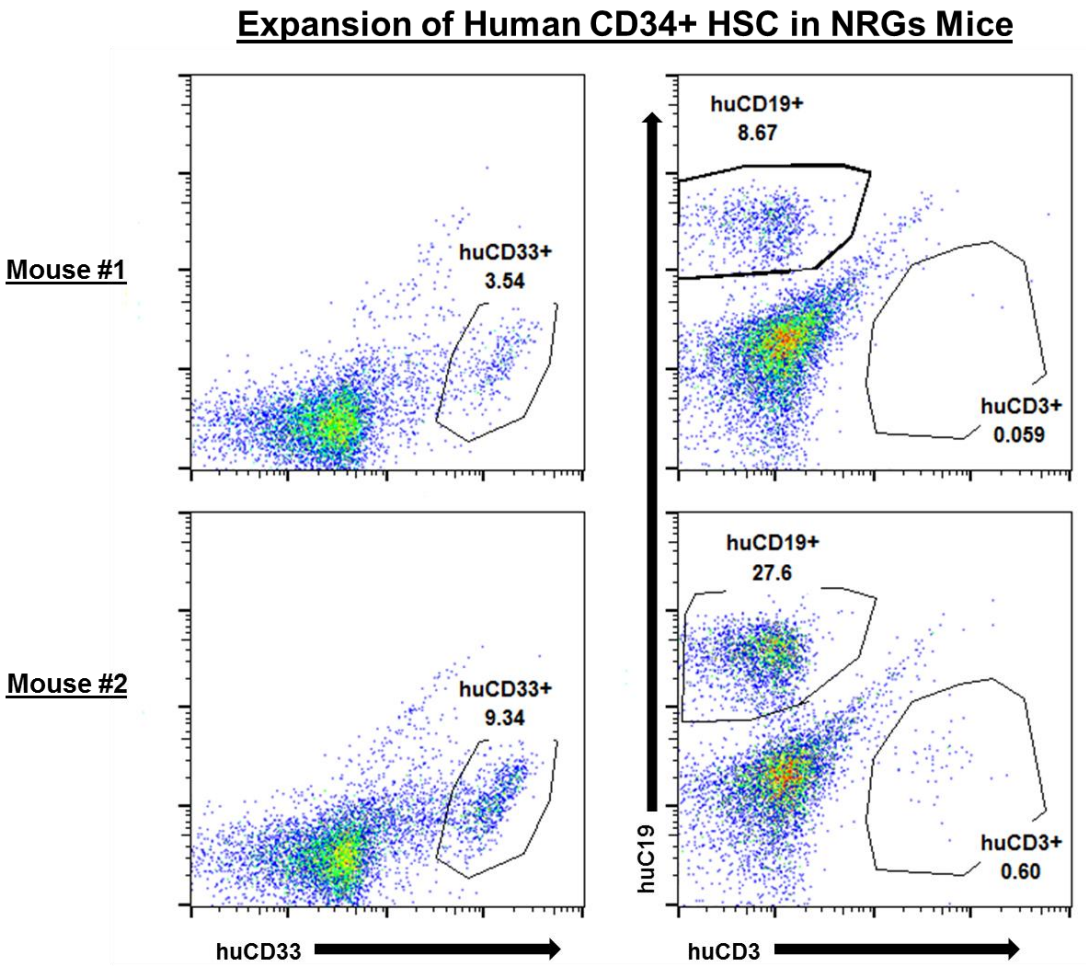

Figure 7: FACS analysis of BM from sub-lethally irradiated NRGS mice engrafted with 5 x 10^5 human CD34+ HSCs. All three mice were sacrificed approximately 100 days post-injection of cells and their spleens and BM stained for the presence of huCD33, huCD19, and huCD3. Only the BM of two of the three mice stained positively and showed detectable expression of the three surface antigens. Mouse #1 showed 3.54% huCD33+, 8.67% huCD19+, and 0.059% huCD3+ cells in the bone marrow, while Mouse #2 had considerably higher engraftment with 9.34% huCD33+, 27.6% huCD19+, and 0.6% huCD3+ cells.

DISCUSSION

As previously emphasized acute myeloid leukemia displays a vast heterogeneity that is exquisitely unique to individual patients, and each case presents widely varied prognoses and drug sensitivities. Therefore investigation of novel therapeutic compounds and treatment strategies for AML that have a high likelihood of success in clinical trials require the development of truly translational mouse models, capable of recapitulating patient specific disease as well as standard of care therapy. Previous studies over the past 50 years have led to the development of immune deficient mouse models that are capable of supporting the growth and proliferation of both established human AML cell lines as well as primary patient samples, and these models have become widespread in the study of AML. However, several major hurdles remain in the development of a truly clinically relevant mouse models of AML namely the lack of human cytokine signaling, lack of a functional immune system, poor engraftment rates of primary patient samples, and finally the inability to tolerate clinically relevant doses of standard of care chemotherapy. Thus the focus of this study was to establish mouse models of AML pathogenesis that could overcome these issues, and more sound methodology for the investigation of novel therapeutics in a patient specific manner.

Our studies hinged on a newly developed strain of mice dubbed NRGS, which expresses three human cytokines and have functional DNA repair, and first we investigated the engraftment of established human AML cell lines (representing distinct subtypes) and subsequent leukemogenisis. Mice were tail vein injected with 1x 10^6 OCI-AML3 or KG1 cells and similar to observed *in vitro* growth rates mice engrafted with OCI-AML3 cells developed leukemia and had to be sacrificed in 30-32 days, while mice injected with KG1 cells had to sacrificed in only 23-25 days. All mice showed distinct signs of leukemic distress including hind limb paralysis as well as breathing difficulties, and necropsy revealed pronounced splenomegaly in all mice. This correlates with growth rates obseverved *in vitro* with KG1 cells showing a much faster growth rate and doubling time as compared to OCI-AML3 cells. Analysis of the spleen and BM showed the presence of human cells in both locations with approximately 50% of the cells in the BM identified as human cells, through human CD45 expression, upon injection of both cell lines. Interestingly OCI-AML3 cells were found at a much higher percentage in the spleen (approximately 30%) as compared to KG1 cells possibly indicating differential preference of location based on AML subtype. Finally both FACS analysis of surface markers and visual morphology of human cells isolated from the BM and/or the spleen confirmed the faithful maintenance of the human phenotype in the mouse microenvironment. These data indicate that NRGS mice allow for the reliable engraftment of human AML cells of varied subtype, and eventually develop fatal human leukemia detectable in the spleen and bone marrow.

After confirming reliable rates of engraftment and consistent leukemic progression using established human cell lines we examined the engraftment of patient derived leukemia, obtained from the spleen of an NSG mouse after tail vein injection of primary patient AML and subsequent sacrifice upon development of leukemia. 1.25 x 10^5 patient AML cells were injected into NRGS mice and 41 days later a humane endpoint was achieved and all mice had to be sacrificed with most displaying either hind limb paralysis or breathing difficulties. This data indicates that NRGS mice show reliable rates of efficient sample engraftment as well as predictable disease progression, both essential to our model system. FACS analysis of human cells obtained upon sacrifice showed continued expression of CD45 and CD11b as compared to initial analysis of the cells upon thawing. However after exposure to the *in vivo* environment and possibly the human cytokines, patient cells harvested from leukemic NRGS mice showed a strong expression of both CD33 as well as CD14, both undetectable upon defrost or short term *in vitro* cell culture. These findings highlight the importance of not only the *in vivo* microenvironment, but also human cytokine signaling, and suggest that they are crucial to accurately maintain the patient specific AML phenotype. In future studies it will be critical to examine the engraftment of primary patient samples taken directly from the clinic as our cells were passaged once through an NSG mouse which may have resulted in selection for cells that were best adapted to the mouse microenvironment. The expression of human cytokines (IL-3, GM-CSF, and SF) is expected to greatly facilitate the implantation and engraftment of primary patient cells, but this remains to be investigated in our hands.

Perhaps the most important goal of this study was to establish a clinically comparable and efficacious dosing regimen of induction therapy using doxorubicin and cytarabine. Based on the work of our collaborator Dr. Jim Mulloy, we tested a "5+3" induction therapy regimen composed of bolus tail vein injections of 3 mg/kg doxorubicin for the first three days and 75 mg/kg cytarabine for five days, which was shown to be well tolerated in NRG mice and comparable to clinically experienced doses of these drugs.[48] Therapy using this dosing regimen was highly efficacious in the treatment of NRGS mice engrafted with our patient derived leukemia and resulted in a significant increase in average survival of 11.4 days. These data are crucial to our model system as we conclusively show that not only can NRGS mice be reliably engrafted with patient derived AML cells and develop leukemia, but also that our "5+3" induction therapy regimen is effective in treating patient derived leukemia and provides a significant survival advantage. Future studies will not only attempt to escalate the dose of induction therapeutics but also use this regimen as a more relevant control during the study of novel therapeutics, alone and/or as combinatorial therapy, against patient derived AML.

A major shortcoming not addressed by this model is the influence of a functional immunity on both leukemic progression and therapeutic efficacy of experimental therapeutics. In an attempt to test our "5+3" induction therapy regimen in a model with a functional immune system we injected 5 x 10^5 spleenocytes from a WT FVB mouse harboring our own in house murine AML, into sub-lethally irradiated WT FVB recipient mice and after a week these mice were put on our "5+3" regimen. This AML is generated by simultaneous retroviral overexpression of MYC and

BCLxL in normal WT FVB BM hematopoetic progenitors. MYC is widely recognized as one of the most potent oncogenes and BCLxL an extremely strong proliferative signal, and overexpression of both in hematopoetic progenitors result in an extremely aggressive and transplantable murine AML. Data obtained from this study clearly indicates that our model induction therapy regimen also provides a significant survival advantage to WT FVB mice transplanted with aggressive MYC/BClxL induced AML, and a fully functional immune system.

Additionally, we attempted to engraft sub-lethally irradiated NRGS mice with normal human CD34+ HSCs to determine if multilineage expansion of human immune cells was possible. While human cells of both the T- and B-lineage were detected in two of the three mice in the study, the rates of differentiation and expansion were generally poor. While disappointing, these results highlight the feasibility of developing NRGS mice with normal functional human immunity in conjunction with patient derived AML. It is likely that conditioning of the mice is required for the engraftment of human HSCs and their subsequent differentiation and expansion into immune cells.

Novel therapeutics and combinatorial therapies, especially immune based therapies, could be tested for efficacy alone and in combination with the standard of care therapy in such a model, and compounds that show efficacy in both models will most likely have a higher likelihood of success in clinical trials. The successful discovery of truly translational novel therapeutics for AML will hinge on the development and implementation of similar, more clinically relevant mouse models of patient specific AML that faithfully recapitulate the unique cytogenetic and

morphological profile of patient disease, the influence of the BM microenvironment, human cytokine signaling, and finally the influence of a functional immune compartment.

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Abstracts

2015	Barve, A., Beverly L. 2015. Exploring novel differentiation therapies for acute myeloid leukemia. Research!Louisville, Louisville, KY.
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