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Characterizing the role of the AML1 -ETO translocation in hematopoietic stem cells.

Cristina G. de Guzman
University of Alabama at Birmingham

CHARACTERIZING THE ROLE OF THE AML1-ETO TRANSLOCATION IN HEMATOPOIETIC STEM CELLS

by

CRISTINA G. DE GUZMAN

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Name of Candidate Cristina G. de Guzman

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A hematopoietic stem cell (HSC) transformed by a genetic mutation or mutations is responsible for the establishment of a leukemic hierarchy. These genetic mutations frequently include chromosomal translocations that have been specifically associated with particular acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) subtypes. The t(8;21)(q22;q22) translocation, which fuses the eight-twenty-one (*ETO*) gene on human chromosome 8 with the acute myelogenous leukemia-1 (*AML1*) gene on chromosome 21 (AML1-ETO), is one of the most frequent cytogenetic abnormalities associated with AML. This translocation is seen in approximately 12 to 15% of AML cases and is present in about 40% of AML cases with a French-American-British classified M2 phenotype. We have generated a murine model of the t(8;21) translocation by retroviral expression of AML1-ETO in purified HSC. Animals reconstituted with AML1-ETO-expressing cells recapitulate the hematopoietic developmental abnormalities seen in the bone marrow of human patients with the t(8;21) translocation. Primitive myeloblasts were increased to approximately 10% of bone marrow by 10 months post-transplant. Consistent with this observation was a 50-fold increase in myeloid colony-forming cells in vitro. Accumulation of late-stage metamyelocytes was also observed in

bone marrow along with an increase in immature eosinophilic myelocytes that showed abnormal basophilic granulation. HSC numbers in the bone marrow of 10-month-post-transplant animals were 29-fold greater than in transplant-matched control mice, suggesting that AML1-ETO expression overrides the normal genetic control of HSC pool size. In summary, AML1-ETO-expressing animals recapitulate many (and perhaps all) of the developmental abnormalities seen in human patients with the t(8;21) translocation, although the animals do not develop leukemia or disseminated disease in peripheral tissues like the liver or spleen. This finding suggests that the principal contribution of AML1-ETO to AML is the inhibition of multiple developmental pathways. Our model has brought new insights into the understanding of the existence and unique properties of the leukemic stem cell that could be exploited for therapeutic interventions. We hope that the methods used to generate this mouse model by retroviral transduction of HSC will be applied to studies of other chromosomal translocations, as well.

DEDICATION

This dissertation is dedicated to my late father, Norberto T. de Guzman, M.D., Ph.D., and my fiancé, David Edward Strong.

My late father supported my decision to join the Department of Medical Genetics and continues to live on in my heart with his philosophy on the success of science and the pursuit of opportunity.

David has driven over 11,000 miles to support my career and has waited so patiently for me to finish. He has been a monumental influence in achieving my goals and aspirations and has encouraged me in the most difficult times.

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LIST OF ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AML1	acute myeloid leukemia-1
AML1-ETO	acute myeloid leukemia-1-eight-twenty-one
C/EBPα	CCAAT-enhancer-binding protein alpha
C/EBPε	CCAAT-enhancer-binding protein epsilon
C/EBP	CCAAT-enhancer-binding protein
CBFβ	core-binding factor β
CCD	cleidocranial dysplasia
cDNA	complementary deoxyribonucleic acid
CFU-GM	granulocyte-macrophage colony-forming units
CSF-1R	colony-stimulating factor-1 receptor
DNAse	deoxyribonulease
DNA	deoxyribonucleic acid
e	embryonic day
EMSA	electromobility shift assay(s)
ENU	*N*-ethyl-*N*-nitrosourea
ETO	eight-twenty-one
FAB	French-American-British

FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FISH	fluorescence in situ hybridization
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDAC	histone deacetylase(s)
HSC	hematopoietic stem cell(s)
IFN	interferon
IgM	immunoglobulin M
IL-3	interleukin-3
IRES	internal ribosomal entry site
LSC	leukemic stem cell(s)
LT-HSC	long-term hematopoietic stem cell(s)
MDS	myelodysplastic syndrome
MPP	multipotent progenitor(s)
mRNA	messenger ribonucleic acid
MSCV	murine stem cell virus
N-CoR	nuclear receptor corepressor
PAX5	gene for B-cell-specific activator protein (BSAP)
pI-pC	polyinosinic-polycytidylic acid
RNAse	ribonuclease
RNA	ribonucleic acid

LIST OF ABBREVIATIONS (Continued)

RT-PCR	reverse-transcriptase polymerase chain reaction
TCR	T-cell receptor(s)
TGFβ	transforming growth factor β

INTRODUCTION

Hematopoietic Stem Cells (HSC). Stem cells are unique and distinct entities within a highly ordered and multilineage cell system capable of self-renewing and/or giving rise to multipotent progenitors (MPP) that differentiate into more mature cells that define particular organs (reviewed in 147). The most well-studied stem cell is the hematopoietic (blood-forming) stem cell. HSC distinguish themselves from other types of blood cells in that they self-renew and function as precursors to their more differentiated erythroid, myeloid, and lymphoid progeny, including red blood cells; platelets; granulocytes; monocytes; and natural killer, B, and T cells.

The discovery of HSC emerged with the studies of Becker et al., Siminovitch et al., Till and McCulloch, Weissman, and Wu et al. in the 1960s where a certain population of bone marrow cells gave rise to myeloid-erythroid colonies in the spleens of lethally irradiated mice (9, 126, 136, 147, 156). These colonies were serially transplanted into secondary mice and gave rise to all hematopoietic lineages. Hematopoietic stem cells were isolated by fluorescence-activated cell sorting (FACS) and monoclonal antibodies and dye-staining that distinguished HSC from other hematopoietic cells (37, 89, 103, 129, 141) and were tested for clonogenicity (28, 127, 139, 151). Two subsets of HSC can be further delineated into long term and short term (86). Long-term hematopoietic stem cells (LT-HSC, Thy-1.1^{lo}Sca-1^{hi}Lin^{-}Mac-1^{-}CD4^{-} of C57/BL/Ka-Thy1.1 mice) can indefinitely self-renew for the lifetime of an individual, whereas short-term HSC (Thy1.1^{lo}

Sca-1^{hi} Lin^{-}Mac-1^{lo}CD4^{-}) are more limited in their self-renewal and can only reconstitute lethally irradiated animals up to 8 weeks. FACS analysis and clonal in vitro assays confirmed a hematopoietic hierarchy: LT-HSC → ST-HSC → MPP → oligopotent progenitors → differentiated progeny (reviewed in 147). MPP give rise to oligopotent progenitors (85) such as the common lymphoid progenitor that only gives rise to natural killer, T, and B cells (59) and the common myeloid progenitor that gives rise to the megakaryocyte-erythroid progenitor and the granulocyte-monocyte progenitor (1). These cells are limited in their self-renewal and are incapable of reversing their differentiative program under normal circumstances (147).

Stem cells that give rise to blood originate from the ventral mesoderm in vertebrates (165). Embryonic or "primitive" hematopoiesis emerges from the extra-embryonic regions of the yolk sac blood islands at embryonic day (e) 7.25 and continues through e12. HSC that are responsible for definitive or "adult" hematopoiesis in the mouse arise from the yolk sac at extremely low frequencies (35, 137) but primarily from the intraembryonic dorsal aorta, genital ridge/gonads, and pro/mesonephros region at 10 (days postcoitum) (72). Recent evidence has indicated that HSC that emerge from the AGM region are of endothelial origin (23). Definitive HSC undergo two successive and distinct waves of migration before birth. The first migration travels from the AGM to the fetal liver at 11 dpc (72). At 16.5 dpc, LT-HSC migrate from the fetal liver to the bone marrow and spleen prior to birth (83), where the bone marrow eventually becomes the primary site for adult hematopoiesis. Cell surface adhesion molecules play an important role in these processes (47).

Extreme fluctuations in HSC frequency within the bone marrow of young, wild-type mice are unusual and suggest that steady-state numbers of HSC are tightly controlled (43, 44). However, the frequencies and numbers of HSC vary across mouse species (88). Two candidate loci that map to mouse chromosome 1 have been found to associate with a quantitative variation in stem cell frequency, suggesting a multigenic control of stem cell frequency. Maintenance of steady-state levels requires instructive mechanisms to dictate whether an HSC will self-renew symmetrically (self-renew HSC) or asymmetrically in order to give rise to more differentiated progeny (self-renew and differentiate HSC) (147). This process involves a delicate balance among entry into cell cycle (14), programmed cell death (apoptosis), and circumvention of a finite replicative potential (Hayflick limit).

Studies of mice treated with bromo-deoxyuridine and analysis of the kinetics of bromo-deoxyuridine incorporation into the cells' DNA have indicated that a majority of HSC are primarily quiescent (G_0) whereby only 8 to 10% of HSC undergo cell cycle per day (14). This finding supports the idea that extrinisic and/or intrinisic mechanisms tightly control the number of HSC in the bone marrow by regulating the number of HSC that actively undergo cell cycle (147). However, by 1 to 3 months, over 99% of HSC have undergone cell cycle (21).

Analysis of mice transgenic for the *Bcl-2* (anti-apoptotic gene) driven by the *H2K* promoter further provides evidence that apoptotic mechanisms play a role in regulating HSC numbers, as well (27). HSC numbers from *Bcl-2* transgenic mice were increased 2.4-fold over that of wild-type mice. Interestingly, the number of HSC in $S/G_2/M$ phase in *Bcl-2* transgenic mice was decreased by 0.6-fold and had a greater advantage in re-

populating the bone marrow when compared to controls, suggesting that cell-autonomous apoptotic mechanisms function to control the number of HSC.

Every cell has a finite replicative potential (reviewed in 41, 45), which can be attributed to the fact that every cell division shortens the telomeres (chromosomal ends) by 50 to 100 bp, eventually leading to the vulnerability of unprotected chromosomes to interchromosomal fusions and, ultimately, cell crisis (22). Self-renewing and cancer cells evade this crisis by upregulating telomerase activity, which catalyzes the addition of hexanucleotide repeats onto the chromosomal ends (18, 84). LT-HSC exhibit high telomerase activity like cancer cells, which suggests a mechanism by which HSC evade finite replicative potential (84).

HSC also possess the unique property of indefinite self-renewal in order to give rise to all hematopoietic cells for the lifetime of the adult (112). However, a longer half-life renders an HSC more vulnerable to acquiring additional mutations that could transform the HSC (112). More differentiated cells would have to acquire this gain-of-function activity, an event that seems less likely to occur given the odds of acquiring additional events for a transformation in addition to self-renewal. Recent studies have posited the hypothesis that HSC serve as the target cell in leukemia for reasons described above (13). We will discuss the initial studies that support the hypothesis of a leukemic stem cell (LSC; 8, 29-31, 38, 54, 140). Before doing so, it is necessary to review acute myeloid leukemia (AML).

AML. AML is seen in approximately 85% of acute leukemia cases in adults and 20% in children and accounts for 1.2% of cancer-related deaths, with an incidence

of 2.2 per 100,000 in the United States every year (107). This incidence increases to 12.6 per 100,000 for patients age 65 and older (65). AML is characterized by the accumulation of large numbers of abnormal hematopoietic cells that fail to differentiate into functional granulocytes or monocytes (32) and is categorized according to the differentiative state of the blast population based on the French-American-British (FAB) classification system. Patients are symptomatically fatigued due to decreased red blood cells and can generally present with hemorrhaging and with fevers and infections that are attributed to decreased platelets and white blood cells, respectively (65). Hepatomegaly, splenomegaly, leukemia cutis, lymphadenopathy, bone pain, and gingival and central nervous system involvement result from leukemic infiltration into any of these tissues. Patients also typically present with granulocytic sarcomas and can also have leukostasis, including ocular and cerebrovascular dysfunction or bleeding from hyperleukocytosis (more than 100,000 white cells/mm^3), and rarely present with hyperuricemia and hypocalcemia.

AML cases are diagnosed based on morphological Wright-Giemsa stains of bone marrow and peripheral blood cells to distinguish the differentiative state of the blast (65). Blasts containing a large nuclear-to-cytoplasmic ratio, round-to-irregular nuclei, distinct nucleoli, and a presence of Auer rods (azurophilic granules within lysosomes) and fine azurophilic granules in the cytoplasm and accounting for greater than 20% in the bone marrow are diagnostic of AML (42, 65). AML can be distinguished from acute lymphoblastic leukemia (ALL) and myelodysplastic syndrome (MDS) based on immunohistochenical and immunological staining for myeloid differentiation and the presence of greater than 20% of myeloblasts. Cytogenetic analysis of

specific chromosomal translocations and myeloperoxidase, nonspecific esterase, and Sudan Black histochemical staining further differentiates specific AML subtypes (FAB M0-M7). The role of specific chromosomal translocations and their associations with certain AML FAB subtypes will be discussed later.

The heterogeneity of AML cases reflected in the various AML subtypes can be explained in two ways (13). First, the leukemogenic event can occur in many cell types in the stem/progenitor hierarchy, thereby altering the normal developmental programs and causing the arrest of a cell at a particular stage. This explanation would predict great variability in LSC/progenitors across all FAB subtypes. The second model proposes that transformational mutations occur in primitive HSC that are only realized at different cell developmental stages depending on the type of transformation. Therefore, variation across LSC from this model would not be seen.

Emergence of the LSC. Griffin and Lowenberg determined that leukemic blasts have a limited proliferative capacity (39). Initial studies in the 1960s to investigate this issue found that in clonal in vitro colony assays on mouse myeloma cells isolated from the ascites, only 1/100 to 1/1,000 cancer cells were capable of forming colonies and that 1 to 4% of these cells could form spleen colony-forming cells in vivo (12, 16, 106, 154). Thus, it was postulated that a primitive stem cell that self-renews and proliferates must be responsible for manifestation of the disease. In other words, an HSC that undergoes a transforming event would seed a leukemic event. Clonal assays on leukemic cells by X-linked polymorphisms and fluorescence in situ hybridization studies have supported the idea of an LSC but have failed to provide solid evidence

dence that these progenitors could proliferate and self-renew in vivo (8, 29-31, 38, 54, 140). The ground-breaking experiments of Bonnet and Dick demonstrated that $CD34^{+}CD38^{-}$ cells from leukemic patients, when transplanted into xenogeneic nonobese diabetic mice with severe combined immunodeficiency were able to proliferate and differentiate into leukemic cells similar to those of patients and to self-renew and expand based on serial transplants (13). This finding supported the model that the leukemic clone is organized as a hierarchy where the transformation occurs at the HSC that self-renews and gives rise to leukemia.

Transforming Events in HSC. Transforming events in cancer cells are defined as defects in a normal cell's regulatory circuitry that renders the cell cancerous (41). If a transforming event via a genetic mutation occurs in an HSC, what type of transforming event or events must occur to give rise to full-blown leukemia? Hanahan and Weinberg propose six essential hallmarks of most if not all cancer cells that are defined as physiological alterations that collectively act through different mechanistic strategies: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (41). HSC already possess a limitless replicative potential, as mentioned previously, a property that is easier to maintain versus a de novo activation (112). This function provides a window for HSC to accumulate other potential transforming events/mutations that can manifest themselves in the HSC if not at the stem cell progeny level.

Chromosomal abnormalities in cancer cells in the 1950s provided the first clues to potential transforming events, although the abnormalities were originally viewed as artifacts rather than factors in the progression of cancer (114). In the 1970s, this idea was challenged with the development of chromosomal banding that specifically identified each chromosome based on its unique banding pattern. Cytogenetic analysis of banded chromosomes detected nonrandom and specific chromosomal breakpoints in different patients with the same disease. This phenomenon suggested a role of chromosomal translocations in cancer progression. Cloning of these translocations led to the discovery of genes that were frequently determined to be transcription factors involved in the regulation of their target genes (114). What were even more interesting were the facts that most of these transcription factors are highly conserved and that many of them recur in various translocations (117).

t(8;21) and Clinical Presentation. The first chromosomal translocation to be identified was t(8;21) (96, 115), fusing the 5' part of the *acute myeloid leukemia-1 (AML1)* gene on chromosome 21 to nearly the entire *eight-twenty-one* (*ETO*) gene on chromosome 8 and creating an in-frame fusion protein, AML1-ETO (80, 82). The fusion protein fuses the first 177 amino acids of AML1 to 574 amino acids of ETO (80). The critical junction of the translocation is conserved on the derivative chromosome 8 (95). Genomic breakpoints usually occur in intron 5 of *AML1* (157) and in introns 1b-2 in *ETO* and cluster within topoisomerase II DNA cleavage and DNase I hypersensitive sites, suggesting a mechanism by which this de novo translocation occurs (163). This translocation is associated with 40% of patients with FABM2 subtype of

AML and accounts for 12 to 15% of all AML cases (65). Some t(8;21) cases have been reported in 6% of FABM1 and rarely in FABM4 and other myeloproliferative syndromes (40). Interestingly, the presence of the *AML1-ETO* transcript by reverse transcriptase-polymerase chain reaction analysis has been detected even without cytogenetic evidence of the t(8;21) (95). Patients with the t(8;21) translocation in FABM2 typically present at a younger age (<35 years), have frequent splenomegaly, and respond well to chemotherapy with high remission rate and long median survival except for those with loss of the sex chromosome (3, 116, 119, 132). A del(9q) is also associated with the t(8;21) (95).

Characteristic cytology of the blasts can predict the existence of the t(8;21) that is usually confirmed by fluorescence in situ hybridization and cytogenetic and/or RT-PCR analysis (3, 11). Leukemic blasts from t(8;21) patients are generally large and are characterized by the existence of prominent Auer rods, strong myeloperoxidase staining, cytoplasmic vacuoles, occasional pseudo-Chedriak granules, and various glass pink inclusions that persist in the maturing leukemic cells (11, 95, 132, 138). Granulocytic dysplasia is also observed with failure of the nucleus to condense and segment at the metamyelocyte stage and myelocyte stage, respectively, that coincides with a rim of basophilia and the presence of the cytoplasm's inability to form granules (salmon-colored) (132). The nucleocytoplasmic maturation in the leukemic cells is asynchronous where the cytoplasm is more differentiated (myeloperoxidase-positive azurophilic granules and peroxidase-negative specific granules) compared to the nucleus, which remains primarily euchromatic and contains a distinct nucleolus (138). Frequent marrow eosinophilia (>5%) is also noted, with poor nuclear maturation and tightly packed

granules that stain dark-blue or deeply purple (characteristic of basophilic granules) in the cytoplasm (64, 132). Other cell lineages are also affected where no monocytic differentiation was observed, as well as a decrease, if not absence, of megakaryocytic elements (11, 132). Minor abnormalities in maturation of erythrocytic precursors were also noted that included mild megablastic change, nuclear fragmentation, and binuclearity (132). The immunological analysis of the t(8;21) cells indicates that these cells express CD19, CD56, and CD34 and are almost negative for CD2 and CD7 (50, 55, 105, 135). The t(8;21) has been associated with a good prognosis and a good response to cytosine arabinoside treatment (131). However, children with t(8;21) respond less favorably (71).

Discovery of AML1 and Runt Family Members. *AML1* (also known in the mouse as *core-binding factor-A2*; *polyoma-enhancer-binding protein 2αB* and, more recently, *Runx1*; 97, 98, 144) was discovered as a result of cloning the *AML1-ETO* translocation (82). This gene maps to chromosome 21q22 in the human, and its murine homologue (*CBFA1*) maps to chromosome 16 in the mouse (98). AML1/core-binding factor (CBFβ) is expressed in the bone marrow, thymus, and peripheral blood (81, 121). AML1 encodes a 453-amino-acid protein (83 KDa) and contains, at its N-terminus, a DNA-binding domain known as the Runt domain (128 amino acids) because of its homology to the *Drosophila melanogaster* pair-rule gene *Runt* (148). The DNA-binding domain of AML1 mediates the association of AML1 to its heterodimeric partner CBFβ, a non-DNA-binding protein (97). CBFβ enhances the binding affinity of AML1 to the cognate DNA-binding sequence, TGT-cGGT, by approximately 10-fold. The DNA core

within the promoters and enhancers of hematopoietic genes such as T-cell receptor (TCR)-α, -γ, -δ, and -β; neutrophil elastase; myeloperoxidase; granzyme B; NP3 defensin; granulocyte-macrophage colony-stimulating factor (GM-CSF); interleukin-3 (IL-3); colony-stimulating factor-1 receptor; and CD11a integrin. This motif may therefore play a role in the expression of these genes (48, 94, 109-111, 123, 133, 144, 145, 150, 164). A proline-enriched serine threonine domain, nuclear matrix targeting signal, and transactivation domain are located downstream from the runt domain (5, 149). Three-dimensional structures have been published on the runt domain (10, 91), the heterodimerization domain (37, 49), and the interaction between AML1 and CBFβ (146). The structure of the Runt domain resembles that of an S-type immunoglobulin fold found on the DNA-binding domains of p53, NF-κB, and STAT proteins (91) and classifies core-binding factor A2 as a member of the p53 family. More recently, the AML1 Runt domain-CBFβ-DNA ternary complex has been described in a 2.6-A resolution in efforts to elucidate the sequence-specific recognition of the consensus sequence, as well as the effects of genetic mutations on the binding of this protein to DNA (15). These molecular structures have provided much information regarding the specificity of certain residues that are implicated in various hematological diseases that will be discussed later.

Low-stringency hybridization of *AML1* to mouse cDNA libraries has led to the isolation of two other *AML* (*Runx*) members, *AML2* (*CBFA3, Runx3, PEBP2aC*) and *AML3* (*CBFA1, PEBP2aA, Runx2*), which share more than 90% of amino acid sequence homology with respect to their Runt domains (6, 148, 153). Like AML1, the other members of the Runt family, AML2 and AML3, are capable of heterodimerizing

with CBFβ and transactivate through the same consensus site. All Runt family members play pivotal roles in development and disease (66).

AML2 is expressed in myeloid and T cells (76, 94) and activated by transforming growth factor β for *immunoglobulin A* class switching (122). Targeted disruption of the *AML2* locus in mice led to an increased proliferation and hyperplasia of gastric epithelial cells (62). Primary cell culture of *AML2*–/– cells demonstrated an insensitivity to TGFβ growth inhibition and decreased apoptosis, thus suggesting a role of AML2 in human gastric cancer. Analysis of human gastric cancer cell specimens confirmed a role of AML2 as a candidate tumor suppressor in gastric cancer development in 30% of the cases (62).

AML3 was initially found in gel-mobility shift assays from the nuclear extracts of Jurkat T cells incubated with the "core" motif from the γ3 site of the TCRγ enhancer (48). The core motifs are critical cis-acting elements in the transcription of *TCRγ* and -*δ* genes as demonstrated by lack of transcriptional activity when mutations of the core motif were introduced (111). These data suggest the importance of the AML3 in the transcription of genes in T-cell development. Surprisingly, knockout studies of *AML3* defined its role as a transcription factor necessary for osteoblast differentiation and bone formation (58). *AML3*–/– mice died just after birth and showed a complete lack of intramembranous and endochondrial ossification due to the maturational arrest of osteoblasts. Heterozygote *AML3*+/– mice exhibited the same skeletal defects seen in patients with human cleidocranial dysplasia (CCD), an autosomal-dominant condition characterized by hypoplasia/aplasia of clavicles, patent fontanelles, supernumery teeth, short stature, and other skeletal changes in growth and patterning, and confirmed the ef-

fect of a mutated *AML3* in CCD (90). Excessive extramedullary hematopoiesis in *AML3*-defi-cient mice was also noted and could be explained by the complete lack of bone marrow formation, thereby forcing the hematopoietic progenitors to seed the liver and spleen (25).

Role of AML1 as a Transcriptional Activator and Repressor. The role of AML1 as a transcriptional activator was ascertained in preliminary in vitro experiments using transient transfection experiments and promoter reporter assays that demonstrated upregulation of the TCRα, -γ, -δ, and -β; neutrophil elastase; myeloperoxidase; granzyme B; NP3 defensin; GM-CSF; IL-3; colony-stimulating factor-1 receptor; and CD11a integrin transcriptional activity as mentioned previously (48, 94, 109-111, 123, 133, 144, 145, 150, 164). Biochemical analysis in myeloid cell lines further revealed that this activating function is synergistically enhanced by the association of AML1 to other hematopoietic-specific, DNA-binding proteins such as the Ets family members, PU.1 and myeloid elf-1-like factor, and CCAAT enhancer-binding protein alpha (C/EBPα) through various interactions between specific domains (70, 108, 150). This finding was further confirmed by the proximity of the DNA-binding motifs of the associated proteins to *AML1* regulatory elements (67). Other associating proteins include p300/CREB-binding protein (56) and Yes-associated protein coactivator protein (130, 158), which serve as a cofactor for Ets members and as a link to Src family kinase-signaling pathways, respectively.

Synergism and cooperation of AML1 is a common theme in B and T cells, as well. Association of AML1 is also demonstrated in developing B cells, where AML1 in-

teracts with the transcription factor, gene for B-cell-specific activator protein (PAX5), through its Runt domain in upregulating *blk* transcription (63). Activation of *TCRα* in T cells is also mediated by AML1 association with alymphoplasia and lymphoid enhancer factor to form a higher-order nucleomultiprotein complex (17).

Conversely, AML1 is also known to act as a transcriptional repressor. AML1-dependent transactivation of the *TCRα* and *-β* enhancers was inhibited by the transducin-like enhancer of split, a mammalian homologue of the *Drosophila* corepressor *Groucho* (51, 61, 68). Inhibition by TLE1 was facilitated by the VWRPY motif in the activation domain of AML1, suggesting a mechanism of AML1 in *TCR* regulation (51, 61). Repression of $p21^{WAF-1}$ expression in NIH3T3 and HEL cells is also mediated by AML1 domains that associate with mSin3A and mSin3B independently of the Groucho interaction motif (68). The mechanism by which AML1 switches from an activator to a repressor is currently being investigated (67). It appears that the function of either one occurs in a context-dependent manner and requires specific interactions between the various domains of AML1 to its associated protein in a cell-specific and promoter-spe-cific manner (67).

AML1–Gene Dosage on Hematopoiesis and Clinical Implications. Knockout studies in mice for *AML1* have determined the critical role of this factor in definitive hematopoiesis. Embryos with homozygous mutations in *AML1* died at e12.5 due to a complete lack of definitive hematopoiesis and intracranial hemorrhaging but had normal primitive yolk-sac-derived erythrocytes (101, 142). It was determined that this hematopoietic defect was intrinsic to the stem cells because *AML1*−/− embryonic stem

cells failed to give rise to hematopoiesis in chimeric animals (101). Knock-out studies of *CBFβ* resulted in the same phenotype, establishing the necessity for CBFβ in AML1 function (120, 143). These experiments suggest a requirement for AML1 in the normal function of HSC or in their development from mesenchymal precursors (92). Attempts to rescue definitive hematopoiesis in *AML1 -/-* embryonic stem cells were successful with the activation domain of AML1 under the endogenous *AML1* regulatory sequences but not with the VWRPY domain known to interact with the Groucho repressor (100). By marking *AML1* expression with the bacterial lacZ marker in the *AML1* locus, North et al. reported the localization of AML1 to definitive hematopoietic progenitor cells and endothelial cells whence HSC are thought to emerge (92). Expression of AML1 was restricted to the endothelial cells present in the yolk sac, the vitelline and umbilical arteries, and the ventral aspect but not the dorsal aspect of the dorsal aorta of the AGM. Thus, it was speculated that AML1 plays a role in the emergence of the HSC from the endothelial cells based on AML1 expression patterns in specific sites from which hematopoiesis occurs (92, 134, 161). This possibility was confirmed in adoptive transfer experiments that demonstrated that all HSC derived from these sites were $AML1^+$ (24, 72, 87, 93).

Efforts to study the role of AML1 in adult hematopoiesis suffer from the embryonic lethality of *AML1 -/-* animals. *AML1* mutations in patients have provided clues to the function of AML1 in the adult hematopoietic system. Screening of various types of leukemia cases documented mutations of the *AML1* gene in 8 of 160 cases (3%) based on RT-PCR and nonisotopic RNase cleavage assays (102). All mutations (silent, heterozygous missense, biallelic nonsense, and frameshift) clustered within the Runt do-

main of AML1. Missense mutations (R80C, K83N, and R177Q) in *AML1* allowed AML1 to heterodimerize with CBFβ but failed to bind to DNA in electromobility shift assays or to transactivate in luciferase reporter assays, respectively. Interestingly, bial-lelic nonsense mutations abolished all AML1 activity and were correlated with the FABM0 phenotype.

Mutations of *AML1* were also seen in patients with the autosomal-dominant familial platelet disorder (thrombocytopenia) with a predisposition to acute myeloid leukemia (77, 128). Linkage analysis identified an 880-kb interval containing the dis-ease gene that cosegregated in six families with FPD/AML. Mutational analysis of candidate genes in this region identified heterozygous *AML1* nonsense mutation and intragenic deletion in four families and two missense mutations in the other two fami-lies. Bone marrow cells isolated from two patients from two independent pedigrees exhibited a decrease in megakaryocyte colony formation, granulocyte-macrophage col-ony-forming units (CFU-GM), and erythroid burst-forming units compared to controls. The authors report a novel finding of haploinsufficiency of AML1 in tumor progres-sion. AML1 haploinsufficiency predisposes patients to acquire AML (77, 128).

Mutations in the *Runt* domain of *AML1* were further identified in blood samples of patients with MDS (2 of 37 cases) (51). The V105ter nonsense mutation failed to heterdimerize with CBFβ, and the R139G missense mutation had a dominant-negative effect on wild-type AML1 in interacting with CBFβ, suggesting that *AML1* mutations may also play a role in progression of MDS to AML. In addition, patients with Down's syndrome experience hematopoietic abnormalities, including increased platelet counts, that could be attributed to increased expression of AML1, which is located in the criti-

cal trisomic 21 region of Down's syndrome patients (7). *AML1* amplification has also been noted in patients diagnosed with B ALL with the t(4;11), t(1;19), and t(12;21) (7).

ETO. Protein sequence of the *ETO* gene (or *MTG8*) has deduced two putative zinc fingers, three proline-rich regions, a proline-enriched serine threonine domain, and several phosphorylation sites in ETO (154). It is homologous to the *Drosophila* homeotic gene, *nervy*, which is expressed in the nervous system (118). The *ETO* gene contains 13 exons and spans 87 kb (155). High mRNA levels of *ETO* have been detected in the nervous system and heart (155). ETO has recently been demonstrated to interact with mSin3 corepressors, the nuclear receptor corepressor (N-CoR), and histone deacetylase (HDAC) (34, 69). Association with either mSin3A or N-CoR by ETO occurs independently, and single point mutations in the *ETO* region that interacts with N-CoR abrogate transcriptional repression by AML1-ETO. ETO and its associated corepressors are unable to bind DNA but are recruited to chromatin through DNA-binding proteins (2, 73). Promyelocytic leukemia zinc finger, a DNA-binding corepressor disrupted in the t(11;17), in acute promyelocytic leukemia, binds to ETO and potentiates repression by promyelocytic leukemia zinc finger (73). Class I HDAC, including HDAC-1, HDAC-2, and HDAC-3, are the only HDAC capable of binding through specific domains in ETO (2). Recently, the function of the *ETO* gene was found to be essential for the gastrointestinal system (20). Homozygous mutants survived until 2 weeks after birth due to growth impairment and massive defects in the gastrointestinal tract.

AML1-ETO–Dominant-Negative Inhibitor of AML1. In vitro studies have determined the dominant-negative effect of AML1-ETO on normal AML1 activity by virtue of its Runt domain and ETO (33, 70, 75, 150). The Runt domain retains its ability to bind to DNA and heterodimerizes with CBFβ and therefore competes with wild-type AML1 (74). ETO is responsible for the repression activity by recruiting N-CoR, mSin3 corepressors, and HDAC to AML1-ETO at promoter sites (69). Furthermore, the *runt* domain and transactivation domain of AML1-ETO contribute to the transcription of *Bcl-2*, an inhibitor of apoptosis, a function not seen in wild-type AML1, and could therefore increase cell survival (57).

Knock-in studies of *AML1-ETO* proved the dominant-negative effect of the translocation on AML1 function. Embryos heterozygous for the *AML1-ETO* allele died at e13.5 due to a lack of fetal liver definitive hematopoiesis similar to the *AML1* homozygous knock-out mice (99, 160). Fetal livers isolated from *AML1-ETO* +/– embryos contained dysplastic multipotent hematopoietic progenitors with high self-renewal capacity in vitro, unlike the *AML1* –/– embryos, where there was a complete absence of HSC activity (101, 142). This phenomenon was confirmed with bone marrow cells transduced with a retrovirus containing *AML1-ETO*, which exhibited increased self-renewal that resembles an immortalized cell line in vitro (99). Transient expression of *AML1-ETO* in zebrafish embryos also resulted in disruption of normal hematopoiesis, aberrant circulation, internal hemorrhaging, and cellular dysplasia and established the zebrafish as another advantageous tool in dissecting the pathophysiology of AML1-ETO (53).

In order to avoid embryonic lethality, an inducible transgene of *AML1-ETO* under the control of tetrocycline-responsive element was activated in the adult mouse by withdrawal of tetracycline from the drinking water (113). In this model, activation of *AML1-ETO* did not result in leukemia over a 24-month period. However, Wright-Giemsa-stained cytospins from serial platings from the bone marrow of activated tet-*AML1-ETO* transgenic mice exhibited more early hematopoietic progenitors and early myeloid cells, with few macrophages in these colonies. Northern blot analysis confirmed the activation of *AML1-ETO* in bone marrow upon tetracycline withdrawal. Another *AML1-ETO* transgenic mouse under the control of the myeloid-specific *MRP8* promoter failed to promote leukemia during the lifespan of the animal, as well (162). However treatment with a strong DNA-alkylating agent, *N*-ethyl-*N*-nitrosourea, induced AML in 55% of mice and acute T lymphoblastic leukemia in 45%. Both models suggest a requirement for secondary mutations to progress to AML. Generation of an interchromosomal translocation between the chrosomomes containing *AML1* and *ETO* genes through Cre-*loxP*-mediated recombination has proved successful, but no findings of hematopoietic effects have been reported (19).

More recently, a mouse strain was developed that contained a conditional *AML1-ETO* knock-in allele with a *loxP*-bracketed transcriptional stop cassette 5' of *AML1-ETO* (*AML1-ETO stop/+*) (46). *AML1-ETO stop/+* mice were crossed against mice containing a transgenic interferon (IFN)-α/β-inducible-*Mx-1* promoter-driven *Cre recombinase* (IFN-α/β-inducible-*Mx1-Cre*). Induction of *Cre* expression was accomplished by endogenous IFNα/β or by intraperitonal injection of a synthetic double-stranded RNA (polyinosinic-polycytidylic acid [pI-pC]) that activates endogenous IFN. AML1-ETO

stop/+/IFN-α/β-inducible-*Mx1-Cre* double transgenic mice treated with pI-pC over a 6-day period had deletion of the stop cassette, resulting in 85% of the bone marrow cells expressing AML1-ETO, even after 2 weeks. Higuchi et al. reported that these bone marrow cells, when plated in methocellulose, primarily gave rise to cytokine-dependent granulocyte-macrophage colonies and rarely to erythoid and mixed lineage colonies compared to controls. Serial platings further assessed the ability of AML1-ETO-expressing cells to bypass culture-induced senescence and establish these cells as permanent cell lines. *AML1-ETO stop*/+/IFN-α/β-inducible-*Mx1-Cre* double transgenic mice treated with pI-pC failed to give rise to leukemia like other previously described AML1-ETO mouse models, even though they had a slight increase in colony-forming-spleen units at day 12, total colony-forming units, and in CFU-GM. Treatment of *AML1-ETO stop*/+/ IFN-α/β-inducible-*Mx1-Cre* double transgenic mice with pI-pC and *N*-ethyl-*N*-nitro-sourea led to development of either granulocytic sarcomas or thymic-derived T-cell lymphoblastic lymphomas in 47% (17/36) of these mice. DNA sequencing screening for mutations in genes commonly mutated in cancers (*p53*, $p19^{ARF}$, $p16^{INK4A}$, *N-ras*, *K-ras*, and *H-ras*) and *AML1* found no genetic abnormalities as cooperating mutations in pI-pC-treated *AML1-ETO stop*/+/IFN-α/β-inducible-*Mx1-Cre* double transgenic mice. The "secondary" mutation appears to render the AML1-ETO-expressing tumor cells growth-factor-independent because no cytokines were required for growth other than fetal bovine serum compared to controls.

Limitations to the Current AML1-ETO Models. To date, all murine AML1-ETO models previously described have advanced our understanding of the effect of

AML1-ETO expression in the hematopoietic system. The dominant-negative activity of AML1-ETO is well documented. The acquisition of additional mutations for full-blown leukemia in AML1-ETO-expressing cells is the most logical explanation for the lack of leukemic onset in these models; however, these models present limitations in addressing the current hypothesis that somatic mutations or, in this case, chromosomal translocations implicated in AML, occur in the HSC. First, the *AML1-ETO*+/− knock-in models have precluded studies in the adult hematopoietic system as a result of embryonic lethality. Second, expression of the AML1-ETO in the inducible models was not clearly and specifically identified in HSC. Activation of the *AML1-ETO* transgene by withdrawal of tetracycline was confirmed by Northern blot analysis on bone marrow cells and not on purified hematopoietic populations such as the HSC. In Western and Northern blot analysis, the *AML1-ETO stop*/+/IFN-α/β-inducible-*Mx1-Cre* double transgenic mice also demonstrated the activation of the *AML1-ETO* transgene upon *Cre* activation by pI-pC. Only cells expressing the IFNα/β receptor would be responsive to *Cre* deletion of the stop cassette and activation of *AML1-ETO*. To date, it has not been established whether any if not all HSC express the IFNα/β receptor. Third, the lack of selectable markers coexpressed with AML1-ETO does not allow one to properly track the development of an AML1-ETO$^+$ cell in vivo.

Could it be possible that development of leukemia requires the expression of AML1-ETO in a specific target cell such as the HSC? This possibility would explain why current models have concluded the lack of a leukemic onset if AML1-ETO was not expressed at the HSC level. Clinical research studies on t(8;21)$^+$ patients in remission support this notion (78, 79). Miyamoto et al. reported persistence of *AML1-ETO*

transcripts in the peripheral blood and bone marrow of patients previously diagnosed with t(8;21)-associated AML and treated with chemotherapy and peripheral blood stem cell transplantation by RT-PCR analysis (78). In another study, FACS-sorted isolation of HSC purified from patients with the t(8;21) in remission also expressed the *AML1-ETO* transcript, which further supports the notion that the somatic mutation must occur in a stem cell but requires additional transforming events that would ultimately lead to AML (79). Bonnet and Dick's model (mentioned previously) also supports the model that a leukemic stem cell serves as the foundation for the establishment of leukemia in animals (13).

Rationale. We hypothesize that the event of a t(8;21) translocation occurs in an HSC, which ultimately leads to development of AML. In order to address this, HSC (Sca-1$^+$c-kit$^+$ Lin$^-$) derived from a congenic *C57B/6-Ly-5.2* mouse strain were transduced with a retrovirus that coexpresses AML1-ETO and the green fluorescent protein (GFP) from an internal ribosomal entry site. AML1-ETO-positive stem cells were re-sorted and transplanted into lethally irradiated congenic Ly-5.1 mice. AML1-ETO/GFP and control GFP animals were monitored for up to 10 months and analyzed for the contribution of AML1-ETO$^+$ cells in the peripheral blood and bone marrow by FACS analysis. Methylcellulose assays for myeloid-progenitor activity and Wright-Giemsa cytospins were performed to characterize the myeloid compartment further. These experiments will enable us to determine whether AML1-ETO causes leukemia and provide answers to the function of AML1 in the hematopoietic system.

HEMATOPOIETIC STEM CELL EXPANSION AND DISTINCT MYELOID DEVELOPMENTAL ABNORMALITIES IN A MURINE MODEL OF THE *AML1-ETO* TRANSLOCATION

by

CRISTINA G. DE GUZMAN, ALAN J. WARREN, ZHENG ZHANG, LARRY GARTLAND, PAUL ERICKSON, HARRY DRABKIN, SCOTT W. HIEBERT, AND CHRISTOPHER A. KLUG

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ABSTRACT

The t(8;21)(q22;q22) translocation, which fuses the *eight-twenty-one* (*ETO*) gene on human chromosome 8 with the *acute myeloid leukemia-1* (*AML1*) gene on chromosome 21 (AML1-ETO), is one of the most frequent cytogenetic abnormalities associated with acute myelogenous leukemia (AML). It is seen in approximately 12 to 15% of AML cases and is present in about 40% of AML cases with a French-American-British classified M2 phenotype. We have generated a murine model of the t(8;21) translocation by retroviral expression of AML1-ETO in purified hematopoietic stem cells (HSC). Animals reconstituted with AML1-ETO-expressing cells recapitulate the hematopoietic developmental abnormalities seen in the bone marrow of human patients with the t(8;21) translocation. Primitive myeloblasts were increased to approximately 10% of bone marrow by 10 months posttransplant. Consistent with this observation was a 50-fold increase in myeloid colony-forming cells in vitro. Accumulation of late-stage metamyelocytes was also observed in bone marrow along with an increase in immature eosinophilic myelocytes that showed abnormal basophilic granulation. HSC numbers in the bone marrow of 10-month-posttransplant animals were 29-fold greater than in transplant-matched control mice, suggesting that AML1-ETO expression overrides the normal genetic control of HSC pool size. In summary, AMLI-ETO-expressing animals recapitulate many (and perhaps all) of the developmental abnormalities seen in human patients with the t(8;21) translocation, although the animals do not develop leukemia or disseminated disease in peripheral tissues like the liver or spleen. This suggests that the principal contribution of AML1-ETO to acute myeloid leukemia is the inhibition of multiple developmental pathways.

INTRODUCTION

The t(8;21)(q22;q22) translocation, which fuses the *eight-twenty-one* (*ETO*) gene on human chromosome 8 with the *acute myeloid leukemia-1* (*AML1*) gene on chromosome 21, is seen in approximately 12 to 15% of acute myelogenous leukemia (AML) cases and in about 40% of AML cases with a French-American-British classified M2 phenotype (10, 27). AML1 also known as Runx1 is a transcription factor with significant homology to the product of the *Drosophila* segmentation gene *Runt* (11, 23). It binds the enhancer core target sequence, TGT/cGGT, in association with a non-DNA-binding subunit, core-binding factor β (CBFβ) (5, 20, 28, 40). Both proteins (together referred to as core-binding factor) interact through the DNA-binding, Runt homology domain of AML1. The inversion (16) disrupts the *CBF*β gene and is found in an additional 12% of AML cases (18). Null mutations in either core-binding factor subunit in mice resulted in embryonic lethality that was associated with intracranial hemorrhaging and a complete absence of definitive hematopoiesis (30, 36, 38, 39).

The t(8;21) translocation fuses the N-terminal 177 amino acids of AML1, which includes the Runt homology domain that binds DNA and interacts with CBFß, in frame with amino acids 30 to 604 of ETO. The fusion protein deletes the C-terminal activation domain of AML1. The *ETO* gene is homologous to the *Drosophila* gene *nervy* and can associate with transcriptional corepressor complexes that include mSin3, histone deacetylases, and nuclear hormone corepressors, which are involved in transcriptional repression (19). Gene knock-in experiments in mice have shown that acute myeloid leukemia-1-eight-twenty-one (AML1-ETO) acts in a dominant-repressive manner to block AML1-dependent transcription (29, 42). Animals heterozygous for an AML1-

ETO knock-in allele displayed a phenotype similar to that of *AML1* or *CBF*β knock-out mice in that they died early in embryonic life (embryonic day 13.5) and exhibited intracranial bleeding and a block in definitive hematopoiesis. One important difference between the knock-out and knock-in phenotypes was the presence of dysplastic hematopoietic progenitor cells within the fetal livers of the knock-in mice that could be readily established as immortalized cell lines in vitro (29).

The consequence of AML1-ETO expression on myeloid lineage development has been explored by using transformed myeloid cell lines that retain some capacity to terminally differentiate. Expression of AML1-ETO in the myeloid cell line 32D.3 inhibits CCAAT-enhancer-binding protein alpha (C/EBPα)-dependent transcription that correlates with a block in granulocytic differentiation in vitro (41). Inhibition of C/EBPα function in these experiments was related to the direct association of AML1-ETO with C/EBPα. Mice that develop in the absence of C/EBPα lack neutrophils and are blocked in granulocytic development at the myeloblast stage (44). Significant downregulation of C/EBPα has also been seen in patient samples bearing the t(8;21) translocation, thus establishing C/EBPα as a potentially critical target gene in AML1-ETO-associated leukemia (31, 32).

Recent efforts to establish animal models for the t(8;21) translocation have involved both transgenic and Cre-Lox-mediated interchromosomal translocation approaches. One study used the tetracycline-OFF system to conditionally activate expression of an AML1-ETO transgene in vivo (35). No abnormal hematopoiesis in peripheral blood or bone marrow was observed over a 2-year time period in AML1-ETO-expressing animals. In vitro colony-forming cell assays showed no difference in the

number or type of colonies that were generated from AML1-ETO or control bone marrow potential during serial passage in methylcellulose. Cells isolated from these platings exhibited an immature myeloid morphology.

A second transgenic approach used a myeloid-specific promoter, *MRP8*, to drive expression of AML1-ETO specifically in the myeloid lineage (43). Again, no abnormal hematopoiesis was seen in the animals in the absence of additional mutations generated by *N*-ethyl-*N*-nitrosourea treatment of newborn mice. Finally, Buchholz et al. used the Cre recombinase system to conditionally activate an interchromosomal AML1-ETO translocation after the onset of definitive hematopoiesis (6). No characterization of hematopoiesis was made in this study.

Morphological and phenotypic analysis of bone marrow from t(8;21) AML patients has revealed a number of characteristic abnormalities in myeloid lineage cells. Large basophilic blasts with a prominent Golgi zone, abnormal granulation, cytoplasmic vacuoles, and a single Auer rod are common (1, 26, 37). Nuclear maturation in granulocytes is generally characterized by abnormal nuclear condensation at the metamyelocyte stage and a failure to segment properly (37). Some patient samples exhibit a marked marrow eosinophilia (>5%) with distinct basophilic granulation (37). In leukemic samples, it is not clear which of these abnormalities are attributable to the activity of AML1-ETO.

In our experiments, we transduced a purified population of hematopoietic stem cells (HSC) with an AML1-ETO-expressing retrovirus that coexpresses the green fluorescent protein (GFP) from an internal ribosome entry site (IRES). HSC that were transduced with the AML1-ETO retrovirus were resorted and then transplanted into lethally

irradiated, congenic animals that differed at the *Ly-5* locus. Our results showed a striking phenotype in both the stem cell compartment and the myeloid cell lineages of all reconstituted AML1-ETO animals. We observed a progressive increase in both the frequency and absolute number of HSC in the bone marrow of AML1-ETO-expressing animals that was 29-fold greater than stem cell numbers in GFP control animals by 10 months posttransplant. The expansion of AML1-ETO-expressing HSC no longer seemed to be restricted by the normal genetic control of HSC pool size (8, 25) but was exhaustible, based on serial transplantation experiments. At 10 months posttransplant, myeloid colony-forming progenitors were expanded approximately 50-fold, which was consistent with increases in the percentage of myeloblasts and promyelocytes to 5 to 14% of total bone marrow. Eosinophil development was also affected, in that we observed a significant increase in immature eosinophil myelocytes that exhibited abnormal basophilic granulation.

In summary, animals that express AML1-ETO in HSC recapitulate many (if not all) of the developmental abnormalities seen in human patients with the t(8;21) translocation but require additional secondary mutations for disease progression to acute myeloid leukemia.

MATERIALS AND METHODS

Generation of retrovirus. *AML1-ETO* was cloned upstream of the IRES element into the *Eco*RI site of the parental murine stem cell virus (MSCV) IRES GFP vec-tor (13). Retroviral constructs were transiently transfected into BOSC23 ecotropic

packaging cells by calcium phosphate coprecipitation (15, 33). Viral supernatants were titrated using NIH 3T3 cells. Titers ranged between 3×10^6 and 1×10^7 IU/ml.

HSC isolation and retroviral transduction. An enriched population of HSC of the surface phenotype Sca-1^+c-Kit^+ Lin^- were isolated by fluorescence-activated cell sorting (FACS) and prestimulated in cytokines as previously described (15). Bone marrow cells from 5-fluorouracil-treated mice (isolated 4 days post-intraperitoneal injection of 150 mg of 5-fluorouracil/kg of body weight) were treated with ACK (0.15 M NH_4Cl and 0.01 M $KHCO_3$) for 5 min on ice to lyse red blood cells and then stimulated for 24 h. After stimulation, cells were cocultured on transiently transfected and irradiated (30 Gy) BOSC23 cells in the presence of 4 μg of Polybrene/ml for 48 h prior to transplantation.

Transplantation. Congenic, C57BL/6-Ly-5.1 (Ly-5.1) mice (3 to 4 months of age) were used as transplant recipients. Prior to transplantation, Ly-5.1 mice were lethally irradiated with 10 Gy in a split dose separated by 3 h. Then 300 to 400 resorted GFP^+/Ly-5.2^+ HSC and a radioprotective dose of 2×10^5 Ly-5.1 bone marrow cells were transplanted into anesthetized mice by retro-orbital injection. A total of 4×10^6 bone marrow cells were used in serial transplant experiments and 1×10^6 to 6×10^6 bone marrow cells were used in 5-fluorouracil transplants. Mice were maintained for 2 to 3 weeks on acidified water containing neomycin sulfate (1.1 g/liter) and polymixin B sulfate (10^6 U/liter) or sulfamethoxazole (400 mg/liter).

Histology. For cytospin preparation, 4×10^4 bone marrow cells in phosphate-buffered saline-12% fetal calf serum (FCS) or methylcellulose colonies in 150 μl of Iscove's modified Dulbecco's medium-12% FCS were centrifuged onto glass slides and stained with Wright-Giemsa. Blood and bone marrow counts were determined manually.

Myeloid colony-forming assay. A total of 1,000 each of AML1-ETO/GFP$^+$ or GFP$^-$ myeloid scatter-gated cells isolated from the same mouse were sorted into Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated FCS and then plated into MethoCult3434 medium (StemCell Technologies) supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF; 0.5 ng/ml; R & D Systems). Colonies were typed at day 10.

Western blot. Approximately 3×10^6 myeloid scatter-gated cells were sorted as either AML1-ETO/GFP$^+$ or GFP$^-$ from two AML1-ETO animals 3 months post-transplant. Cells were lysed in Laemmli buffer and run on a 10% polyacrylamide gel. AML-ETO was detected with a rabbit polyclonal antibody raised against a peptide encoding residues 32 to 50 of the human AML1 protein (11). The primary staining was visualized with a goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate se-condary antibody and enhanced chemiluminescence (Amersham Pharmacia).

Northern blot. Total RNA from approximately 8×10^6 myeloid scatter-gated cells was isolated with RNA Stat-60 according to the manufacturer's instructions (Tel-test B, Inc., Friendswood, Tex.). Total RNA (7.5 μg) was run on a 1% agarose-0.6%

formaldehyde gel, transferred to a Hybond-N (Amersham) membrane, and hybridized according to the supplier's protocol. A murine glyceraldehyde-3-phosphate dehydrogenase (Ambion) and C/EBPα probe (kindly provided by Dan Tenen, Harvard University) were used for detection.

RESULTS

Generation of a murine model of the t(8;21) translocation. HSC of the phenotype c-Kit$^+$Sca-1$^+$Lin$^-$ (Fig. 1B) were double-sorted to a purity of >98% and then transduced with retroviral supernatant containing either the control or AML1-ETO vector (Fig. 1A). Each vector was derived from MSCV and contained an IRES to allow coexpression of GFP. Transduction efficiencies ranged from 20 to 28% for the AML1-ETO virus and 30 to 40% for the control virus (Fig. 1C). Transduced HSC isolated from C57BL/6-Ly-5.2 mice were resorted for GFP expression and then transplanted into lethally irradiated, congenic C57BL/6-Ly-5.1 animals at a dose of approximately 300 GFP$^+$ cells per recipient. AML1-ETO-expressing animals were also generated by transplanting retrovirally transduced whole bone marrow cells isolated from 5-fluorouracil-treated animals (see Materials and Methods).

Expression of AML1-ETO from the retroviral vector was confirmed by Western blot analysis with a polyclonal anti-AML1 antibody and GFP$^+$ myeloid lineage cells sorted from the bone marrow of an 8-week-postreconstitution AML1-ETO animal (Fig. 1D). The anti-AML1 antibody was raised against a peptide encoding residues 32 to 50 of the human AML1 protein (11). The immunizing peptide has a three- amino-acid dif- ference from the murine and human sequence, so a direct comparison between

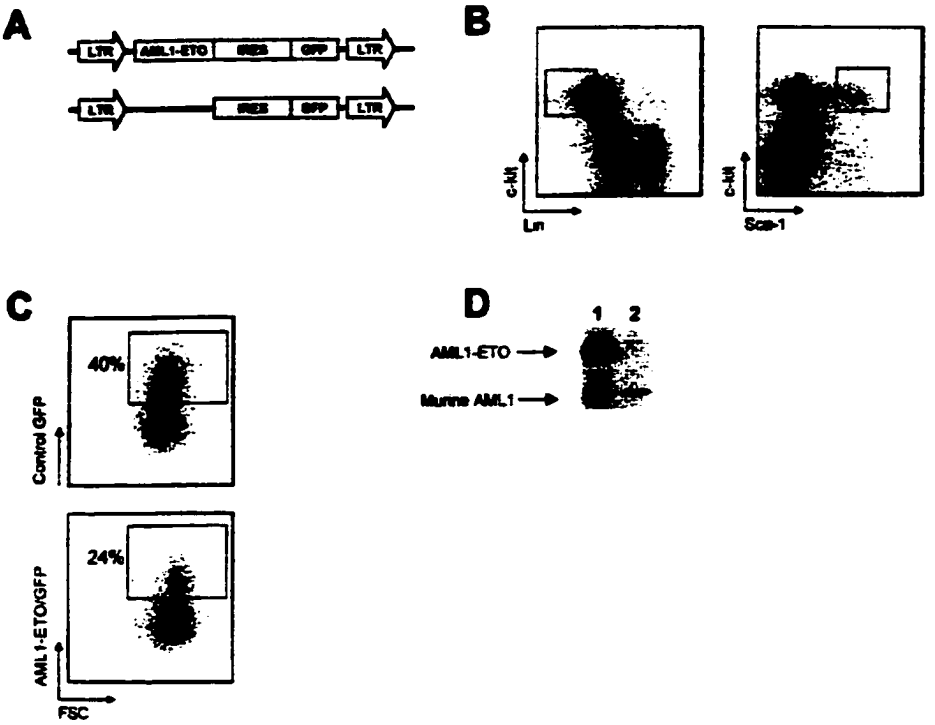


FIG. 1. Retroviral transduction of murine hematopoietic stem cells. (A) Schematic diagram of MSCV retroviral constructs (control MSCV IRES GFP and MSCV *AML1-ETO* IRES GFP). (B) Gating used for sorting the HSC phenotype c-Kit$^+$Sca-1$^+$Lin$^-$ (where Lin represents a cocktail of antibodies to the mature blood cell antigens Mac-1, Gr-1, Ter119, B220, CD3, CD4, CD5, and CD8). (C) Flow cytometric analysis of HSC 24 h after retroviral transduction. Approximately 300 Ly-5.2$^+$ HSC from control or AML1-ETO transductions were transplanted with a radioprotective dose of 2×10^5 Ly-5.1$^+$ whole bone marrow cells into each Ly-5.1$^+$ recipient animal. (D) Wes-tern blot analysis of GFP$^+$ (lane 1) or GFP$^-$ (lane 2) myeloid scatter-gated cells FACS-sorted from the bone marrow of an 8-week-posttransplant AML1-ETO animal probed with a polyclonal anti-AML1 antibody.

the levels of retrovirally expressed AML1-ETO and endogenous AML1 protein in myeloid lineage cells is not possible. Western blot analysis with lysates from 2.5 million FACS- sorted AML1-ETO-expressing myeloid cells and 2.5 million Kasumi-1 cells, which represent a human AML cell line that expresses the t(8;21) translocation, showed that Kasumi-1 cells expressed AML1-ETO at twofold-higher levels than retrovirally expressed AML1-ETO in primary cells (data not shown).

Abnormal myelopoiesis and decreased B lymphopoiesis in AML1-ETO/GFP$^+$ peripheral blood cells. The effect of the AML1-ETO fusion protein on hematopoiesis was monitored in AML1-ETO-expressing and control GFP animals by FACS analysis of peripheral blood. All AML1-ETO (n = 29) and control GFP (n = 26) recipients were reconstituted with up to 85% of peripheral blood cells expressing the Ly-5.2 donor marker (Fig. 2A). Donor cells that silenced expression of the GFP marker were present in all reconstituted animals (15). Peripheral blood myeloid cells were analyzed by costaining with Mac-1 (CD11b) and Gr-1. AML1-ETO/GFP$^+$ cells showed an abnormal Mac-1/Gr-1 phenotype in all AML1-ETO mice compared to control GFP mice or to non-AML1-ETO-expressing cells (GFP$^-$) within the AML1-ETO mice (Fig. 2B).

Notably absent in the AML1-ETO/GFP$^+$ population was a subset of Mac-1^{lo} Gr-1^{hi} cells that represents an essentially pure population of mature neutrophils (17). In addition, there was an overrepresentation of a unique subset of cells that expressed high levels of Mac-1 and intermediate levels of Gr-1. This subset of cells was present in the peripheral blood of all AML1-ETO mice at all time points analyzed and did not increase in frequency between 2 and 10 months posttransplant (n = 3 for animals analyzed up to

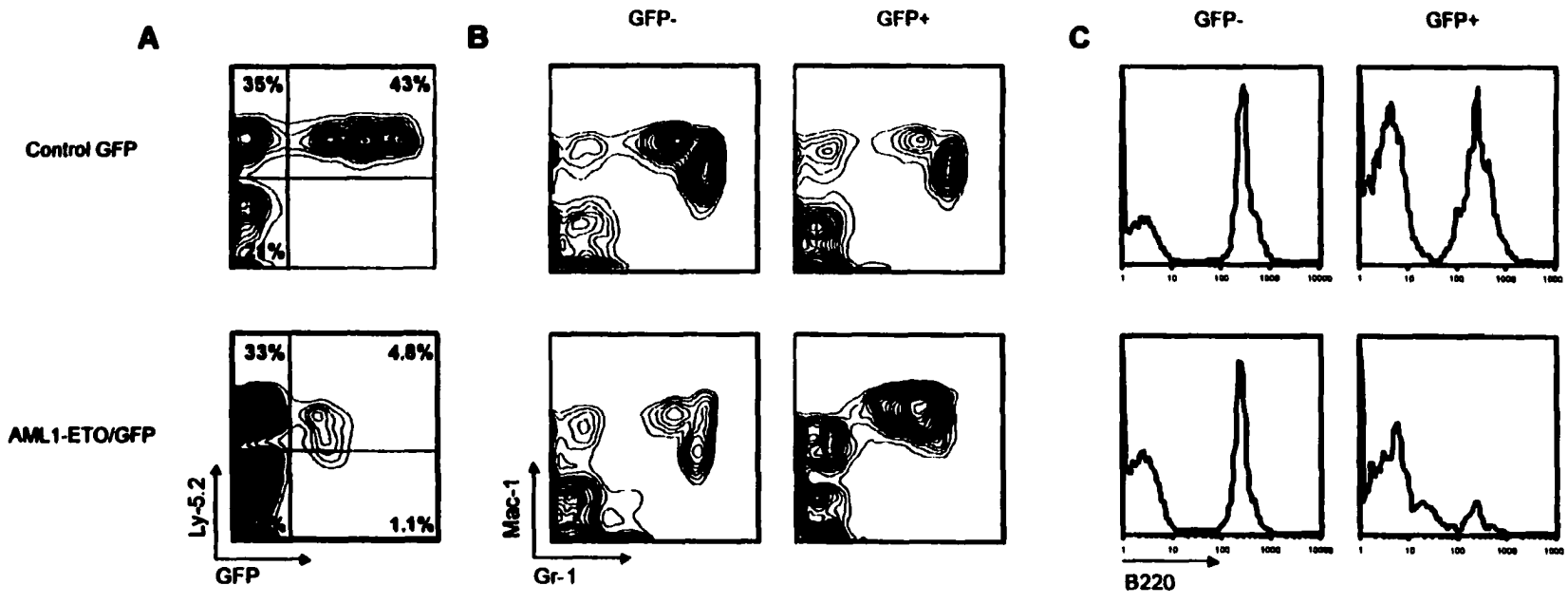


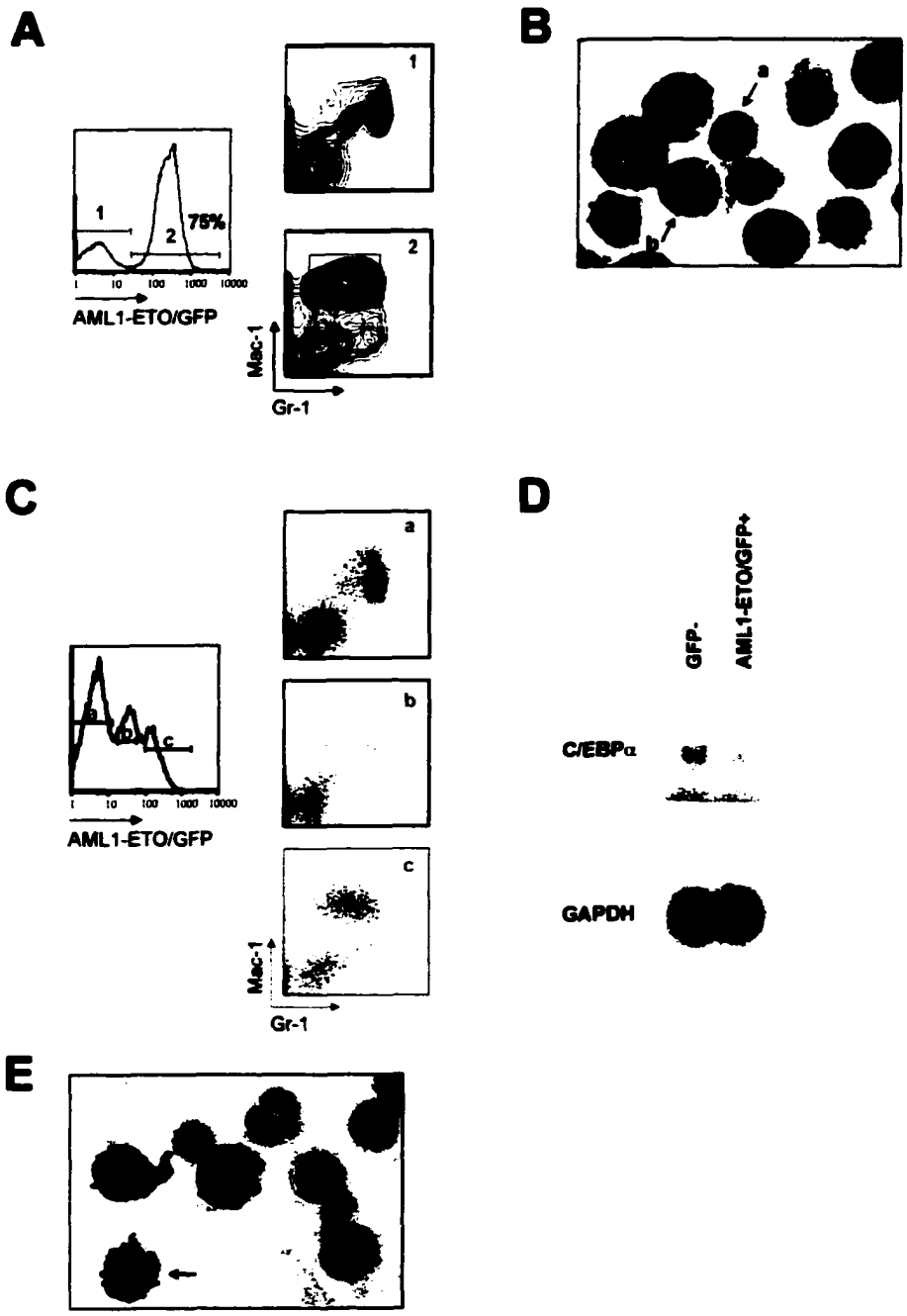
FIG. 2. Abnormal myelopoiesis and decreased B lymphopoiesis in AML1-ETO/GFP$^+$ peripheral blood cells. (A) Flow cytometric analysis of peripheral blood cells from animals at 2.5 months posttransplantation stained with an antibody to the Ly-5.2 donor marker. Peripheral blood cells were gated as GFP$^-$ or GFP$^+$ and analyzed for (B) simultaneous Mac-1 and Gr-1 or (C) B220 expression. Note the reduction in Mac-1^{lo}Gr-1^{hi} cells that represent mature neutrophils and an overrepresentation of Mac-1^{hi}Gr-1^{int} cells in the AML1-ETO/GFP$^+$ population compared to the GFP$^+$ control. FACS plots are representative of all cocultured whole bone marrow transplants of control GFP (n = 21) and AML1-ETO-expressing (n = 26) mice and all purified HSC transplants of control GFP (n = 5) and AML1-ETO-expressing (n = 3) mice.

10 months and $n = 26$ for animals analyzed between 1 and 6 months posttransplant). In all analyses, the GFP^- cells within the AML1-ETO mice resembled the GFP^- and GFP^+ cell profiles from control animals.

Peripheral lymphoid cells in transplant recipients were analyzed by staining for B220 and CD3 expression on B and T cells, respectively. Analysis of the $B220^+$ population in AML1-ETO and control GFP mice showed that B220 expression was significantly lower in AML1-ETO/GFP^+ cells compared to controls (Fig. 2C). At this point, it is not clear whether the $B220^{lo}$ cells represent an immature B-cell population in the periphery or simply downregulation of B220 expression on mature B cells. The number of cells expressing CD3 was dramatically decreased in AML1-ETO/GFP^+ cells, al-though this observation was also seen in some of the control GFP^+ animals, making it difficult to draw definitive conclusions on the role of AML1-ETO in T-cell development at this point (data not shown).

Abnormal myelopoiesis in AML1-ETO-expressing bone marrow cells. Given the abnormal myeloid phenotype in AML1-ETO/GFP^+ peripheral blood cells, AML1-ETO-expressing mice were sacrificed to further investigate myeloid cell development in the bone marrow. AML1-ETO mice were sacrificed at 10 months posttransplant and analyzed for myeloid cell abnormalities by Mac-1/Gr-1 staining. All AML1-ETO mice ($n = 3$) exhibited the same Mac-1^{hi}Gr-1^{int} population in the majority of AML1-ETO/GFP^+ bone marrow cells compared to GFP^- control myeloid cells analyzed from the same bone marrow (Fig. 3A). The appearance of this abnormal population in bone marrow was dependent on the level of AML1-ETO expression, as dem-

FIG. 3. Abnormal myelopoiesis in AML1-ETO-expressing bone marrow cells. Flow cytometric analysis of bone marrow from a 10-month-posttransplant AML1-ETO mouse. (A) Bone marrow cells were gated on (panel 1) GFP^- and (panel 2) AML1-ETO/GFP^+ bone marrow cells and analyzed for expression of Mac-1 and Gr-1. The data are representative of all AML1-ETO-transplanted animals between 2 and 10 months posttransplant. The Mac-1/Gr-1 profile in panel 1 is identical to what is seen in bone marrow from control GFP animals. (B) Wright-Giemsa-stained cytospin preparation of AML1-ETO/GFP^+, Mac-1^{hi}Gr-1^{int} cells gated as shown in panel A (×100 magnification). Arrows indicate (a) a banded neutrophil and (b) a metamyelocyte. (C) Graded levels of AML1-ETO expression show distinct Mac-1/Gr-1 phenotypes in bone marrow. (D) Northern blot analysis of RNA isolated from GFP^- and AML1-ETO/GFP^+ bone marrow cells from a 3-month-posttransplant AML1-ETO animal. The blot was probed with a 3' fragment of the *C/EBPα* cDNA and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Quantitation of transcript levels was done on a phosphoimager. (E) Wright-Giemsa staining of an eosinophil myelocyte, showing abnormal basophilic granulation from the bone marrow of a 10-month-posttransplant AML1-ETO animal.



onstrated by an AML1-ETO-expressing mouse that expressed both low and high levels of GFP (Fig. 3C). The dose-dependent phenotype in the myeloid lineage was not unexpected because AML1-ETO functions as a dominant inhibitor of normal AML1 activity (21, 29, 42).

In order to determine the morphology and function of the cells residing in the Mac-1^{hi}Gr-1^{int} population, we sorted these cells for Wright-Giemsa staining and for assays of myeloid colony-forming potential in methylcellulose. We observed no myeloid colony-forming activity in the Mac-1^{hi}Gr-1^{int} population when 2,000 of these cells were plated in triplicate in methylcellulose over a 10-day in vitro culture period (data not shown). Plating 1,000 GFP^- control myeloid lineage cells gave rise to 1 to 10 myeloid colonies (see below). Wright-Giemsa cytospin preparations indicated that 95% of the Mac-1^{hi}Gr-1^{int} cells were metamyelocytes and immature band-form neutrophils (Fig. 3B), which is consistent with the lack of myeloid colony-forming activity in the population. In addition, there were no observed myeloblasts or promyelocytes in counts of 1,000 Mac-1^{hi} Gr-1^{int} cells from 10 independent microscope fields from two animals.

In the animal shown in Fig. 3A, 38% of the total marrow was comprised of this myeloid subset. The other animals analyzed had 8 and 14% Mac-1^{hi}Gr-1^{int} cells in the bone marrow at 10 months posttransplant. Interestingly, morphological characterization of bone marrow from human patients with the t(8;21) translocation also showed abnormal nuclear condensation at the metamyelocyte stage (37).

Recent studies have demonstrated that AML1-ETO downregulates transcription of *C/EBPα*, a transcription factor necessary for granulocytic differentiation, in patients with t(8;21)-associated leukemia (31). To determine whether C/EBPα expression was

affected in AML1-ETO/ GFP$^+$ cells, RNA was isolated from FACS-sorted myeloid AML1-ETO/GFP$^+$ and myeloid GFP$^-$ cells from the same AML1-ETO-expressing animal. Northern analysis showed that the level of *C/EBPα* mRNA expression in AML1-ETO-expressing cells was 2.5-fold lower than in GFP$^-$ myeloid lineage cells (Fig. 3D). These results confirm that AML1-ETO expression causes a downregulation of C/EBPα levels in myeloid lineage cells.

Increased myeloid progenitors in the presence of AML1-ETO. Changes in the number of myeloid progenitors in bone marrow were determined by in vitro colony-forming cell assays with GFP$^+$ and GFP$^-$ cells isolated from AML1-ETO mice at 2 and 10 months posttransplant. One thousand myeloid scatter-gated AML1-ETO/GFP$^+$ or GFP$^-$ cells from the same animal were sorted and then cultured in methylcellulose for 10 days (Fig. 4A). AML1-ETO/GFP$^+$ cells isolated from 2-month-posttransplant animals ($n = 3$) gave rise to large colonies with an average of 16 myeloid colonies per 1,000 cells plated in triplicate, compared to somewhat smaller colonies observed in GFP$^-$ cell platings, which averaged 4 myeloid colonies per 1,000 cells plated (Fig. 4B). The fourfold increase in progenitor numbers compared to controls was statistically significant ($P < 0.001$). The expansion of myeloid progenitors was further increased in the bone marrow of 10-month-posttransplant animals ($n = 3$), where 1,000 AML1-ETO/GFP$^+$ cells gave rise to an average of 48 myeloid colonies, compared to an average of 1 myeloid colony in GFP$^-$ control cells (Fig. 4B). The percentages of total myeloid cells in bone marrow (GFP$^+$ and GFP$^-$) were 58, 41, and 72% from the three AML1-

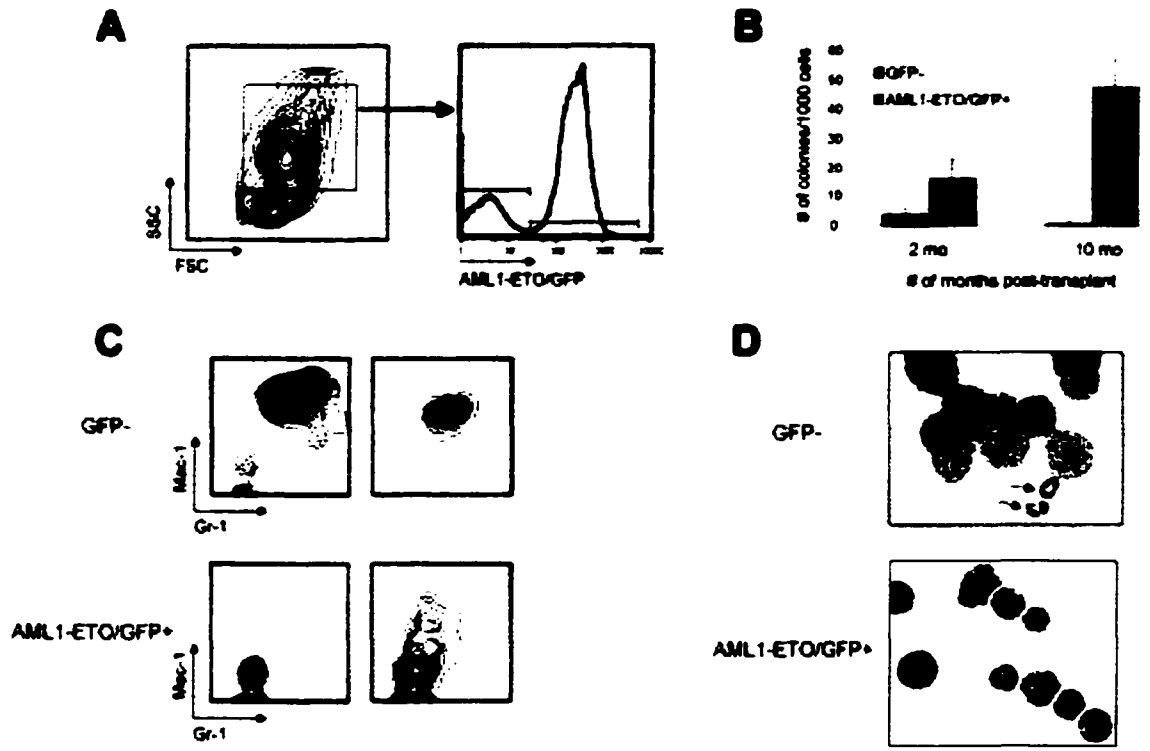


FIG. 4. Increase in myeloid colony-forming cells in AML1-ETO animals. (A) Myeloid scatter-gated cells were sorted as GFP$^-$ or AML1-ETO/GFP$^+$ from a 10-month-old AML1-ETO mouse, and 1,000 cells from each population were plated in triplicate into M3434 methylcellulose medium (10 ng of murine recombinant interleukin-3, 10 ng of human recombinant interleukin-6, and 50 ng of murine recombinant stem cell factor per ml) supplemented with 0.5 ng of GM-CSF; R & D Systems per ml. (B) Three independent AML1-ETO animals at 2 and 10 months posttransplant were used in the analysis. Colonies were enumerated (1 colony of >200 cells) and characterized 10 days after plating. (C) Representative FACS plots of individual methylcellulose colonies stained with Mac-1 and Gr-1. Two representative plots are shown for each sample. (D) Cytospin preparations of GFP$^-$ and AML1-ETO/GFP$^+$ colonies stained with Wright-Giemsa. Arrows indicate mature, segmented neutrophils among the GFP$^-$ cells that were not seen in any AML1-ETO-expressing colonies.

ETO 10-month animals. The percentages of GFP^+ myeloid cells in the same animals were 44, 46, and 91%, respectively. This indicates that there was not preferential expansion of GFP^+ myeloid lineage cells in these animals (except in the latter case) even though the frequencies of specific myeloid subpopulations were significantly altered in cells that expressed AML1-ETO.

Wright-Giemsa-stained cytospins of colonies derived from AML1-ETO/GFP^+ cell platings showed a mixed lineage phenotype that included immature myeloid cells and mature macrophages (Fig. 4D). There were no segmented neutrophils present in AML1-ET0-expressing colonies. In contrast, cytospin preparations of GFP^- colonies showed a number of mature segmented neutrophils (arrows in Fig. 4D). FACS analysis of individual colonies stained with Mac-1 and Gr-1 confirmed that GFP^- colonies were almost completely differentiated (9 of 10 colonies were Mac-1^+Gr-1^+). In contrast, AML1-ETO/GFP^+ colonies remained primarily undifferentiated, with negative or low-level expression of Mac-1 in only a fraction of the cells from a single colony (Fig. 4C).

To assess the percentages of myeloid cell types in the bone marrow of the three animals used for methylcellulose assays at 10 months posttransplant, myeloid-gated GFP^+ and GFP^- cells were cytospun and stained with Wright-Giemsa. The three AML1-ETO/GFP^+ fractions of marrow were highly shifted in representation toward primitive myeloid cell types, with 17, 48, and 21% myeloblast/promyelocytes, compared to 1, 3, and 3%, respectively, of the same cell subsets in the GFP^- controls (Table 1). Overall, the frequency of myeloblast/promyelocytes in bone marrow of the three AML1-ETO animals (after normalization for the total percentage of GFP^+ myeloid

TABLE 1. Differential counts of sorted myeloid bone marrow cells from 10-month-posttransplant AML1-ETO animals[a]

	% of >300 cells/sample					
GFP expression	Blasts + pro	Mye	Meta + band	Baso	Mature eosino	Eosino myelo
Yes	17	12	69	<1	<1	2
	48	7	44	<1	<1	1
	21	6	73	<1	<1	<1
No	1	7	89	<1	3	<1
	3	1	92	<1	4	<1
	3	8	89	<1	<1	<1

[a] Blasts + pro, myeloblasts and promyelocytes; Mye, myelocytes; Meta + band, metamyelocytes and band nuclear granulocytes; Baso, basophils; Mature eosino, mature eosinophils; Eosino myelo, eosinophilic myelocytes.

Statistical analysis (*t* test) showed statistically significant differences between GFP^- and AML1-ETO/GFP^+cells in myeloblasts and promyelocytes ($P \leq 0.05$), metamyelocytes and band nuclear granulocytes ($P < 0.04$), and mature eosinophils ($P \leq 0.01$).

cells) was 4.6, 9.5, and 14.0%. These results support the data from the in vitro colony-forming cell assays, indicating that a substantial increase in myeloid progenitor populations had occurred by 10 months posttransplant in the AML1-ETO animals.

One criterion used in the characterization of AML in humans is the presence of greater than 20% myeloblasts in bone marrow (12). Although the percentage of myeloblasts/promyelocytes in the 10-month-posttransplant AML1-ETO animals was not 20%, the results clearly indicate that a highly abnormal condition exists in the myeloid lineage that becomes more pronounced over time. The lack of leukemia in the AML1-ETO animals was further supported by bone sections characterized at 4 months posttransplant, which did not show evidence of granulocytic foci. This was also true of the spleen and liver at this stage (data not shown). Finally, there was a significant increase in immature eosinophil myelocytes that exhibited abnormal basophilic granulation in AML1-ETO-expressing animals at both early and late times posttransplant (one at 2 months, one at 3 months, and three at 10 months; Fig. 3E and Table 1). A similar abnormality in eosinophil development is also seen in human patients with the t(8;21) translocation (37).

Expansion of HSC in AML1-ETO-expressing mice. In order to characterize the HSC compartment in reconstituted animals, we performed five-color FACS analysis of bone marrow isolated from animals transplanted with cells expressing either the AML1-ETO or control GFP vector (Fig. 5). HSC in reconstituted animals have the same cell surface phenotype (c-Kit$^+$Sca-1$^+$Lin$^-$) as HSC isolated from unmanipulated

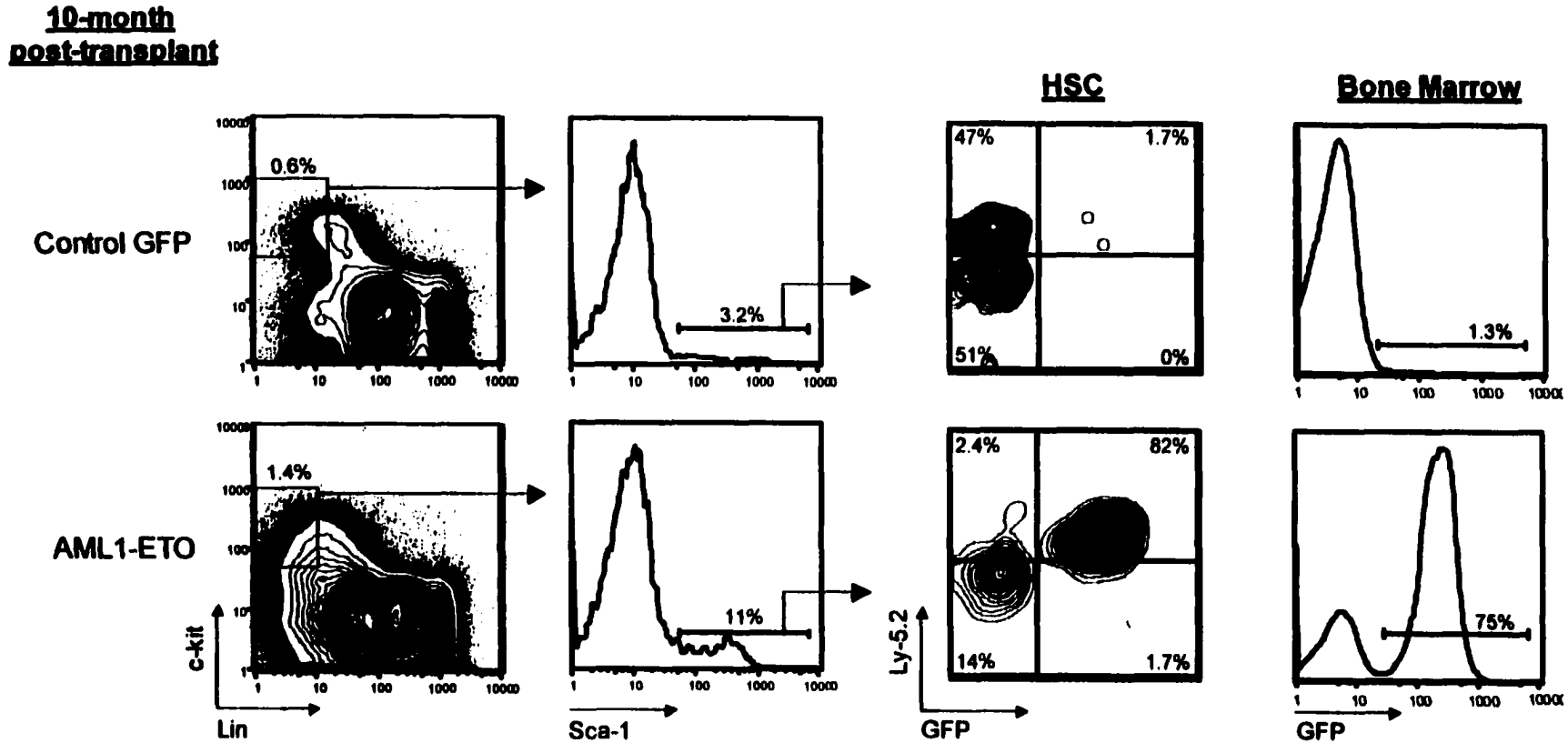


FIG. 5. Expansion of hematopoietic stem cells in AML1-ETO mice. HSC analysis from a 10-month-posttransplant AML1-ETO mouse. Bone marrow cells were stained with c-Kit, lineage marker antibodies (see text), Sca-1, and the Ly-5.2 donor marker. The percentages of cells in individual gated populations are indicated.

bone marrow (24). Bone marrow cells isolated from the tibias and femurs were quantitatively harvested and counted prior to staining to determine absolute HSC numbers. FACS analysis was performed at 2 and 10 months posttransplant of purified HSC and at 2.5 months posttransplant of transduced whole bone marrow cells isolated from 5-fluorouracil-treated animals (Table 2). The latter samples were analyzed to determine whether HSC expansion and absolute number would be influenced by the presence of approximately 10^6 bone marrow cells that were cotransduced and injected with HSC.

Figure 5 shows a representative analysis and gating of one AML1-ETO and one control GFP animal analyzed at 10 months posttransplant. Table 2 summarizes the results from eight AML1-ETO and eight control animals analyzed at the indicated time points. There was a modest expansion (threefold) in the absolute number of phenotypically defined HSC in AML1-ETO-expressing animals at 2 months posttransplant and a dramatic expansion (29-fold) by 10 months. One animal at 10 months had more than 50 times the expected number of c-Kit$^+$Sca-1$^+$Lin$^-$ cells. HSC from AML1-ETO animals transplanted with cocultured whole bone marrow cells were expanded 9.3-fold compared to control GFP animals at 2.5 months posttransplant. At every time point analyzed, the lowest number of HSC in an AML1-ETO animal was higher than the highest HSC number in any of the control GFP animals (Table 2).

The absolute number and frequency of HSC in control GFP animals were highly consistent in all animals, which suggests that the genetic control of hematopoietic stem cell pool size was maintained in primary transplant recipients expressing the control vector (8, 25). In contrast, AML1-ETO-expressing HSC no longer seemed to be restricted by the regulatory mechanisms that influence homeostasis within the stem

TABLE 2. Absolute number and frequency of hematopoietic stem cells in transplanted animals[a]

	AML1-ETO/GFP mice		Control GFP mice		
Time posttransplant (mo)	Absolute HSC no.	Freq. of HSC (%)	Absolute HSC no.	Freq. of HSC (%)	Average expansion (fold)
2	7,755	0.017	1,509	0.004	
	10,802	0.018	2,326	0.007	3
			5,895	0.015	
10	93,960	0.116	6,162	0.011	
	118,556	0.163	10,200	0.020	29
	505,200	0.800			
2.5[b]	52,100	0.146	10,980	0.015	
	16,480	0.032	10,890	0.015	9
	259,980	0.619	13,530	0.022	

[a] Hematopoietic stem cells were derived from the femurs and tibias of transplanted mice. Average expansion is a multiple of the average number of HSC in AML1-ETO-transplanted animals over the average number in control GFP-transplanted animals at a given time point.

bAnimals from whole bone marrow transduction.

cell compartment. Consistent with this speculation was the observation that the increase in HSC number in the AML1-ETO animals was due to an expansion of AML1-ETO/GFP$^+$HSC within the HSC compartment. The percentages of AML1-ETO/GFP$^+$ HSC in the total HSC compartment ranged from 72 to 99% in seven of eight AML1-ETO animals (one AML1-ETO animal had 44% GFP$^+$ HSC), with a mean percentage of GFP$^+$ HSC of 82% (n = 8). This was in contrast to control GFP animals, in which the mean percentage of GFP$^+$ HSC was 15% (n = 8). GFP$^-$ donor (Ly-5.2$^+$) and recipient (Ly-5.2$^-$)-type HSC were present in all animals.

Delayed differentiation in AML1-ETO-expressing hematopoietic stem cells. Despite the high percentage of AML1-ETO/GFP$^+$ HSC at 2 months posttransplant (75 and 81%, n = 2), the percentage of AML1-ETO/GFP$^-$ cells in the bone marrow was only 3.5 and 3.4%, respectively (Fig. 6). In the control GFP animals, the percentage of GFP$^+$ HSC more closely approximated the GFP percentage in the bone marrow. The delayed appearance of more differentiated GFP$^+$ cells in bone marrow was consistent with a delay in the appearance of GFP$^+$ peripheral blood cells in animals transplanted with AML1-ETO-transduced HSC (n = 5 for AML1-ETO; data not shown). In addition, AML1-ETO-expressing HSC were unable to radioprotect lethally irradiated recipient animals at a dose of 600 cells (n = 6), whereas the same dose of control HSC radioprotected and reconstituted four of five animals (data not shown). This supports the notion that AML1-ETO-expressing HSC show a reduced ability to differentiate and an enhanced tendency to undergo cell division events that favor self-renewal. In spite of an apparent partial block in differentiation at 2 months posttrans-

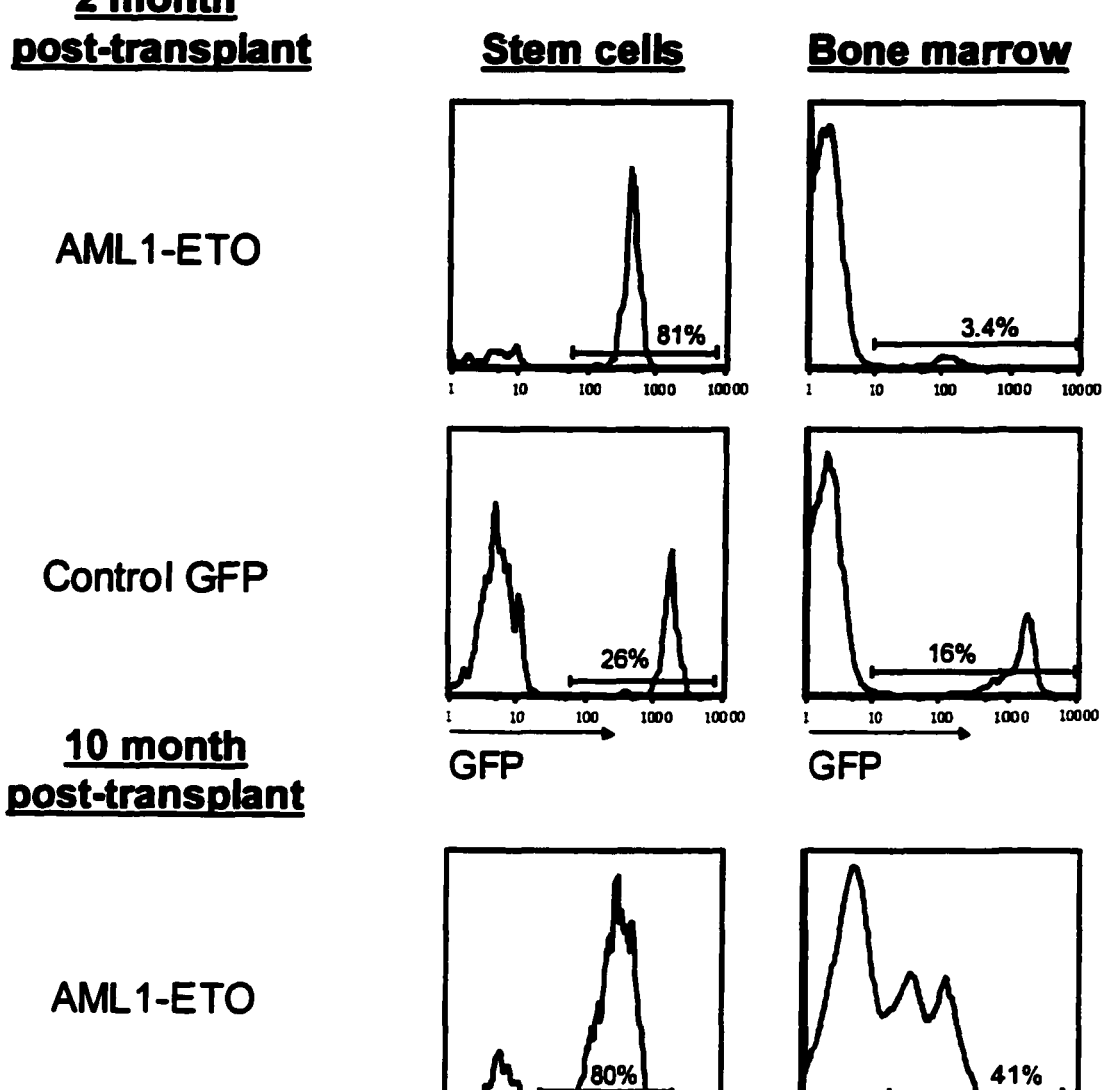


FIG. 6. Delayed differentiation in AML1-ETO-expressing stem cells. The percentage of AML1-ETO-expressing (GFP^+) cells in the stem cell population and in whole bone marrow was contrasted at early (2 months, $n = 3$) and late (10 months, $n = 3$) times postreconstitution. The ratio of GFP^+ cells in the stem cell compartment and in the bone marrow of control GFP animals was similar to the ratio seen in older AML1-ETO animals.

plant, the percentage of GFP^+ cells in older AML1-ETO-expressing animals increased to proportions seen in controls (Fig. 6), which was largely due to an accumulation of GFP^+ myeloid lineage cells.

Maintenance of abnormal myelopoiesis is dependent on sustained expression of AML1-ETO in HSC. The lack of leukemia in AML1-ETO-expressing animals by 10 months posttransplant suggests that secondary mutations or additional time is necessary for disease progression. In an attempt to accelerate a disease phenotype, 4×10^6 bone marrow cells from primary transplant recipients at either 2 or 10 months posttransplant were serially transplanted into multiple secondary recipient animals. Interestingly, only one of four secondary recipients were reconstituted in bone marrow with AML1-ETO/GFP^+ cells at 5 weeks posttransplant with marrow isolated from a 2-month primary donor, even though the bone marrow inoculum would have contained approximately 600 GFP^+ HSC and about 114,000 GFP^+ myeloid lineage cells (Fig. 7 and Table 3). Of the 600 GFP^+ HSC, 60 would be expected to rehome to the

TABLE 3. Serial transplantation of AML1-ETO bone marrow[a]

Time posttransplant (mo)	Primary recpt	Secondary recpt	% GFP^+ HSC	Absolute no. of GFP^+ HSC	% GFP^+ WBM
2	A		75.0	5,816	3.4
1		A1	0	0	0
1		A2	0	0	0
1		A3	97.2	347,976	1.8
1		A4	0	0	0
10	E		97.3	491,559	75.4
4		E1	ND	ND	35
4		E2	ND	ND	18.1
6		E3	69.8	5,641	11.8
2		E4	86.9	21,134	37.2

[a]recpt, recipient. Secondary recipients each received 4×10^6 whole bone marrow cells (WBM) from the primary recipient. ND, not determined.

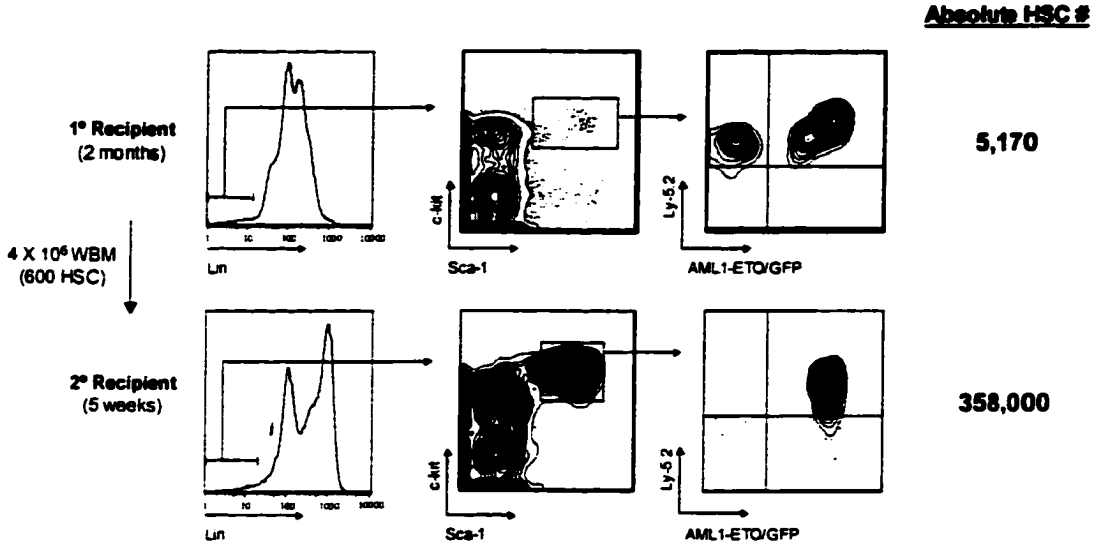


FIG. 7. AML1-ETO expression in stem cells is required for maintenance of abnormal myelopoiesis. Bone marrow from one primary recipient AML1-ETO animal was serially transplanted at a dose of 4×10^6 cells into each of four lethally irradiated secondary mice. Flow cytometric analysis of HSC in one of four secondary animals is shown at 5 weeks posttransplant. All secondary transplant animals received 114,000 AML1-ETO-expressing myeloid cells along with approximately 600 AML1-ETO/GFP$^+$ HSC in the bone marrow inoculum. WBM, whole bone marrow.

bone marrow and approximately 12 would rehome to the tibias and femurs, which represent about 20% of the total marrow cellularity.

The three negative animals all showed high donor reconstitution and no GFP^+ HSC, suggesting that donor GFP^- HSC may have outcompeted GFP^+ HSC during engraftment or that GFP^+ HSC homed less efficiently to marrow than GFP^- HSC. The one animal that was donor reconstituted with AML1-ETO/GFP^+ cells showed an enormous expansion of the HSC phenotype (from a predicted 12 HSC to 358,000 GFP^+ HSC in both tibias and femurs in 5 weeks; Fig. 7). Approximately 33% of the total GFP^+ cells in the marrow of this secondary recipient were c-Kit^+Sca-1^+Lin^-, supporting the observation that AML1-ETO-expressing HSC are partially blocked in their ability to differentiate. Of note was the lack of abnormal myelopoiesis in the absence of AML1-ETO/GFP^+ HSC in the three negative secondary animals. This suggests that the 114,000 coinjected AML1-ETO/GFP^+ myeloid lineage cells do not expand extensively and retain a relatively short half-life in vivo.

Four secondary recipients derived from injection of 4×10^6 bone marrow cells from a 10-month primary transplant animal were all highly reconstituted with AML1-ETO/GFP^+ cells in peripheral blood for up to 6 months posttransplant (Table 3). One animal that was sacrificed at 2 months posttransplant had 21,134 total HSC, which represented a modest 33-fold expansion in HSC number over the 2-month reconstitution period. This was in contrast to the 30,000-fold expansion in 5 weeks seen in secondary recipient A3 (Table 3). The observation that four of four animals were highly reconstituted with AML1-ETO/GFP^+ cells from a 10-month primary donor and only one of four secondary animals was reconstituted with the same number of bone marrow

cells isolated from a 2-month donor may be related to the predicted number of GFP^+ HSC in the inocula. The GFP^+ HSC number from the 10-month donor was approximately 32,000 cells, which was in contrast to the 600 GFP^+ HSC from the 2-month primary donor.

The total expansion of AML1-ETO/GFP^+ HSC in vivo also seemed to be limited by some uncharacterized mechanism. This conclusion is based on the observation that HSC expansion was more severely limited with bone marrow from primary animals that already displayed substantial HSC expansion (Table 3). This may indicate that the genetic mechanisms regulating the replicative life span of HSC are distinct from those that control the steady-state number of stem cells in vivo.

DISCUSSION

Myeloid developmental abnormalities associated with AML1-ETO expression. We have generated a murine model of the developmental defects observed in human patients with t(8;21)-associated AML by retroviral expression of the AML1-ETO fusion protein in HSC. The developmental abnormalities seen in AML1-ETO-express-ing myeloid lineage cells were evident at three levels. First, more differentiated metamyelocytes and band-form neutrophils exhibiting a Mac-1^{hi}Gr-1^{int} phenotype accumulate in bone marrow and become the predominant cell population by 10 months posttransplant (Fig. 3). These cells are also evident in the periphery and are diagnostic for the presence of an 8;21 translocation in bone marrow long before the marrow becomes highly abnormal. However, the Mac-1^{hi}Gr-1^{int} cells in the periphery do not accumulate to the same extent as in bone marrow, suggesting that some developmental

cue necessary for emigration of these cells from the marrow may be absent or that these cells turn over rapidly in the periphery. It remains possible that this maturational cue may involve a nuclear condensation abnormality in metamyelocytes, which is seen in t(8;21) AML.

A second developmental abnormality in the myeloid lineage was seen in bone marrow eosinophils. We observed a unique population of immature eosinophil myelocytes in three of three AML1-ETO animals at 10 months posttransplant that were not observed in non-AML1-ETO-expressing myeloid cells (Table 1). Immature eosinophils that exhibited basophilic granules were also detected at early times posttransplant (one at 2 months and one at 3 months), which indicates that AML1-ETO interferes with normal eosinophil development. Marrow eosinophilia is frequently observed in human AML M2 patients with the t(8;21) translocation, where the eosinophils also have developmental abnormalities associated with abnormal nuclear maturation and basophilic granulation (37).

Finally, early myeloid progenitor cells that give rise to myeloid colonies in methylcellulose expand approximately 50-fold over non-AML1-ETO-expressing myeloid progenitors by 10 months posttransplant. These cells have a reduced ability to differentiate, as evidenced by the immature morphology and cell surface antigen profile of mye-loid colonies in vitro (Fig. 4), which may contribute to their accumulation in bone marrow. In AML1-ETO mice, myeloblasts and promyelocytes increased to between 5 and 14% of total marrow at 10 months posttransplant, which indicates that myelopoiesis within the bone marrow has become highly abnormal but not leukemic at this

stage. Consistent with this interpretation was the absence of Auer rods in the myeloblasts, which are typically seen in human leukemic blasts.

Hematopoietic stem cell expansion in AML1-ETO-expressing animals. The substantial increase in the absolute number and frequency of c-Kit$^+$Sca-1$^+$Lin$^-$ cells in AML1-ETO-expressing animals (Table 2) suggests that AML1-ETO can override the normal genetic control of HSC pool size in mice (8, 25). Interestingly, the absolute expansion of AML1-ETO-expressing HSC was exhaustible in vivo, based on serial transplantation experiments. We observed no AML1-ETO/GFP$^+$ cells in the bone marrow of seven tertiary transplant mice reconstituted with bone marrow from the highly reconstituted secondary recipient animal shown in Fig. 7 (data not shown). These results suggest that AML1-ETO may decrease the proliferative capacity or homing efficiency of HSC in the context of a transplant assay. Consistent with this interpretation is the observation that AML1 regulates expression of certain integrin genes that may be inhibited by AML1-ETO (34).

In addition, AML1-ETO expression in myeloid cell lines was associated with decreased proliferation and a block in myeloid development (7). The increase in HSC numbers in AML1-ETO animals was gradual (Table 2, 2 months) and was associated with an apparent partial block in their ability to differentiate (Fig. 6). This again suggests that proliferation kinetics in the HSC population was not increased by AML1-ETO expression and that HSC accumulation may be related to an enhanced self-renewal potential in the presence of AML1-ETO. It also remains possible that AML1-ETO may enhance HSC survival and thereby increase HSC numbers in vivo. Studies

by Domen et al. have shown that expression of the antiapoptosis gene *bcl-2* in HSC resulted in a 2.4-fold increase in HSC numbers in vivo compared with nontransgenic control littermates (9). However, from this result, it would seem unlikely that blocking cell death would be the only mechanism responsible for the magnitude of HSC increase that we observed in the AML1-ETO-expressing animals. This issue is currently being explored. In any case, the large expansion in the HSC pool size would presumably allow a much larger population of cells that could acquire additional mutations, leading to a more aggressive phenotype resembling AML.

Secondary mutations are required for disease progression associated with t(8;21). The large increase in the frequency of myeloid progenitor cells within the bone marrow of AML1-ETO animals occurred over a 10-month period (Fig. 3), which indicates that disease progression associated with an 8;21 translocation is rather slow. Se-condary mutations that have been associated with AML with an M2 phenotype and the t(8;21) translocation include activating mutations in the tyrosine kinase receptors c-*KIT* and *FLT3* and in the *RAS* proto-oncogene (2, 3, 16). Activating mutations in *FLT3* associated with an internal tandem duplication of the juxtamembrane domain are seen in about 25% of AML cases and 9% of AML cases with an M2 phenotype and t(8;21) (16). Approximately 20 to 30% of AML cases have mutations in the N-*ras* or K-*ras* gene, which are not usually seen in association with the *FLT3* mutations (14). Interestingly, the principal secondary mutations associated with the t(8;21) translocation would all provide a potent mitogenic stimulus that could rapidly expand the abnormal developmental stages that we observed in the AML1-ETO animals.

Animal models of the 8;21 translocation. Other animal models of the t(8;21) translocation have been generated as previously described (see the introduction). Our results are entirely consistent with the results from other studies with the exception that we observed a striking phenotype in the bone marrow of AML1-ETO-expressing animals in the absence of any secondary mutations induced by chemical mutagenesis. From our studies, we can make four observations that may help to resolve the apparent discrepancies in the animal models. First, it is clear that the level of AML1-ETO expression must be high enough to titrate out the activity of both wild-type *AML1* alleles in order to see any phenotype in transgenic or retroviral models (Fig. 3C). Second, it is important that bone marrow characterization be done at early and late times postreconstitution, given the slow nature of disease progression that we observed both in the HSC compartment and within the myeloid lineages (Fig. 4B and Table 2). Third, AML1-ETO must be expressed in the HSC compartment in order to sustain abnormal myelopoiesis in the bone marrow based on serial transplantation experiments (4) (Fig. 7 and Table 3). Finally, disease progression may be accelerated in the retroviral model due to an increased number of HSC that express the t(8;21) translocation at the beginning of the experiment. If very few HSC express AML1-ETO, as might be the case in the inducible transgenic models, we would expect a longer incubation period before highly abnormal conditions exist in the bone marrow.

Currently, all models (with the exception of Cre-Lox-mediated interchromosomal translocation [6]) suffer from the limitations that expression levels of AML1-ETO may not parallel those seen in human t(8;21) patients, and the temporal pattern of expression in hematopoietic cells may also differ. With respect to the latter point, we

did observe AML1 expression in primitive HSC, suggesting that an 8;21 translocation in this population would be expressed (22; C. de Guzman and C. Klug, unpublished data). One distinct advantage of the retroviral system over other approaches has been the ability to identify AML1-ETO-expressing cells with GFP as a surrogate marker for AML1-ETO. This has allowed us to study the abnormalities associated with AML1-ETO expression in animals that exhibit very low percentages of cells that are AML1-ETO/GFP$^+$.

The generation of a murine model of the t(8;21) translocation is significant in that the unique contribution of AML1-ETO to leukemia and developmental dysfunction can be studied at great depth at both the cellular and molecular levels. The animals will also be valuable as tools to explore therapies that specifically target cells that express AML1-ETO and provide a means to address the secondary mutations that are required for disease progression.

ACKNOWLEDGMENTS

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IMPAIRED LYMPHOID DEVELOPMENT IN A MOUSE MODEL OF THE *AML1-ETO* TRANSLOCATION

by

CRISTINA G. DE GUZMAN, ROSE KO, AND CHRISTOPHER A. KLUG

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ABSTRACT

The t(8;21) translocation (acute myeloid leukemia-1-eight-twenty-one [AML1-ETO]) is one of the most frequent chromosomal translocations in acute myeloid leukemia. We recently reported distinct myeloid developmental abnormalities similar to patients and a partial block and expansion of AML1-ETO$^+$ hematopoietic stem cells (HSC) and myeloid progenitors in mice that were transplanted with HSC transduced with an AML1-ETO retrovirus. Very few AML1-ETO$^+$/B220$^+$ and no AML1-ETO$^+$/CD3$^+$ were observed in the peripheral blood of these mice. In order to determine whether B- and T-cell development was inhibited by AML1-ETO, early B- and T-cell progenitors were examined in the bone marrow and thymus, respectively, for AML1-ETO$^+$ cells in these populations. AML1-ETO/green fluorescent protein (GFP)$^+$ cells failed to give rise to CD3$^+$ T cells and expressed low levels of T-cell receptor β (TCR)β in the periphery but appeared to give rise to few SP CD4 and CD8 cells in the thymus and spleen. The decreased frequency of AML1-ETO/GFP$^+$ cells in the thymus further suggests that AML1-ETO may inhibit proper T-cell development, which was confirmed by an expansion of AML1-ETO/GFP$^+$ cells at the TN2 (CD44$^+$ CD25$^+$) stage. No AML1-ETO/ green fluorescent protein (GFP)$^+$ CD19$^+$ cells were observed in all AML1-ETO mice compared to controls, suggesting a block in B-cell commitment. This block correlates with our observation that AML1-ETO/GFP$^+$ B220$^+$ cells were mostly CD43$^+$immunoglobulin M$^-$, which denotes an apparent block in the pre-pro-B-cell to pro-B-cell stages. These studies provide clues to why patients with the t(8;21) translocation fail to give rise to AML1-ETO$^+$ T cells and are immunocompromised in some cases.

INTRODUCTION

The t(8;21) translocation, which encodes the acute myeloid leukemia-1-eight-twenty-one (AML1-ETO) fusion protein, is one of the most frequent chromosomal translocations in acute myeloid leukemia (AML), accounting for 12 to 15% of AML cases and up to 40% of the French-American-British (FAB)M2 AML subtype (11). Other chromosomal translocations involving the *acute myeloid leukemia-1* (*AML1*) gene include the t(3;21) (*AML1-MDS-EVI*) seen in secondary AML and therapy-associated leukemia (17) and the t(11;21) (*TEL-AML1*) found in childhood acute lymphoblastic leukemia, making *AML1* the most commonly translocated gene in human acute leukemia (17).

AML1, also known as core-binding factor A2, Runx1, and polyoma-enhancer-binding protein α2b, is a transcription factor that is homologous to the *Drosophila melanogaster* segmentation *Runt* gene (19, 20, 32) and binds to the DNA sequence TGT/cGGT found in the promoters and enhancers of various hematopoietic genes such as colony-stimulating factor-1, granulocyte-macrophage colony-stimulating factor, myeloperoxidase, neutrophil elastase, granzyme B, CD11a integrin, NP-3 defensin, and T-cell receptors (TCR; 8, 16, 23-25, 28-29, 32-34, 37). AML1 heterodimerizes with its partner core-binding factor β (CBFβ; 20), which has also been implicated in the inv(16) in FABM4 AML leukemia (2). The t(8;21) translocation fuses the N-terminal portion of AML1 containing the runt domain to the transactivation domain of eight-twenty-one (ETO, also known as MTG8; 15) which has been shown to interact with various corepressors, including mSin3A, histone deacetylase, and nuclear receptor corepressor(1, 12, 13).

Knock-out studies in mice have determined the requirement for *AML1* in definitive hematopoiesis, where *AML1*−/− mice are embryonic lethal at embryonic day (e) 13.5 due to the lack of definitive hematopoiesis and intracranial hemorrhaging (22, 30). *CBFβ*−/− mice also demonstrated the same phenotype, further confirming the role for both of these heterodimeric partners in hematopoiesis (27, 31). The dominant-negative role of AML1-ETO on wild-type AML1 was determined in in vitro studies, where overexpression of *AML1-ETO* in 32Dcl3 cell line abrogated terminal granulocytic differentiation (34). This phenotype was further confirmed in *AML-ETO* knock-in mice, where heterozygous *AML1-ETO*+/− mice were embryonic lethal at e13.5 due to lack of definitive hematopoiesis and intracranial hemorrhaging (21, 35) but with an establishment of dysplastic hematopoietic progenitors that could readily be established as immortalized cell lines (21).

Studies on an inducible form of *AML1-ETO* using the tetracycline-OFF system (26) and the myeloid-specific expression of *AML1-ETO* in mice to evade embryonic lethality (36) showed a requirement for a "second hit" to establish a full onset of AML. This finding was confirmed by an inducible *loxP-AML1-ETO* system under the interferon-*Cre*, which demonstrated progression to leukemia upon *N*-ethyl-*N*-nitrosurea treatment to generate additional "second hit"mutations (7). T-cell development appeared to be affected in these mice because no AML1-ETO$^+$ cells were found in the thymus upon activation of its expression. It is likely that AML1-ETO expression could affect the lymphoid compartment because reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of patients with the t(8;21) in remission fails to express AML1-ETO transcripts in T cells and in low levels in B (B220) cells (14).

We previously reported the lack of or downregulation of B220 cells in AML1-ETO-transplanted animals (3). Analysis of T cells also suggested an effect of AML1-ETO on T-cell development, where no AML1-ETO/$CD3^+$ cell was observed in any of our mice. However, the lack of consistency and early posttransplant time points in our controls has precluded our ability to comment on whether T-cell development is actually impaired. In order to determine whether AML1-ETO affects early B- and T-cell development, AML1-ETO^+ cells were analyzed for various B- and T-cell markers that correspond to specific B- and T-cell stages. Our fluoresence-activated cell sorting (FACS) analysis suggests that AML1-ETO expression leads to few mature CD4 and CD8 T cells, which could be due to the accumulation of $CD44^+CD25^+$ (TN2) cells. This suggests a possible block in TCRβ rearrangement. AML1-ETO also blocked B-cell development because no AML1-ETO/$CD19^+$ cells were observed, a finding consistent with an increase in early B cell progenitors (pre-pro-B through pro-B). These results suggest an additional role of AML1-ETO in impairing proper B- and T-cell development and may explain the immunosuppressive phenotype seen in patients with the t(8;21) translocation.

RESULTS

Absence of mature CD3/AML1-ETO/GFP^+ T cells in AML1-ETO recipient mice. AML1-ETO recipients in our previous study suggested a block in T-cell development because AML1-ETO/green fluorescent protein $(GFP)^+$ peripheral blood cells were $CD3^-$ at 1 month posttransplant (3). Due to inconsistencies in $CD3^+$ expression in control GFP mice at 1 month posttransplant, FACS analysis of AML1-ETO and

control GFP mice were performed at least 5 months posttransplant to confirm this observation and allow for mature T cells to migrate to the periphery after transplant. At 8 months posttransplant, AML1-ETO/GFP$^+$ hematopoietic stem cells (HSC) failed to give rise to CD3$^+$ peripheral blood cells compared to controls (Fig. 1A). Cells expressing low levels of AML1-ETO/GFP had a few CD3$^+$ cells, indicating a gene dosage effect (data not shown). Low levels of TCRβ were detected in AML1-ETO/GFP$^+$ cells (Fig. 1B). The frequencies of SP CD4 and SP CD8 were also decreased among AML1-ETO/GFP$^+$ cells compared to controls (Fig. 1C). These data confirmed our conclusion that AML1-ETO expression at the HSC level affects mature T-cell development.

Impaired AML1-ETO/GFP$^+$ thymic T-cell development in AML1-ETO recipients. Despite the fact that there were few AML1-ETO/GFP$^+$CD3$^+$ peripheral blood cells in AML1-ETO recipients, it is likely that the defect in T-cell development occurs after an AML1-ETO stem cell commits to the T-cell lineage. In the thymi of AML1-ETO HSC and 5-fluorouracil transplants, the frequency of cells expressing AML1-ETO/GFP$^+$ was threefold lower than that of control GFP (Fig. 2A), indicating an impairment of AML1-ETO stem cells to populate the thymus compared to controls or a block in thymocyte development in AML1-ETO-expressing cells. FACS analysis of these thymic AML1-ETO/GFP$^+$ cells indicated that AML1-ETO expression can give rise to mature CD4 and CD8 T cells but at a lower frequency compared to controls (Fig. 2B). It is also interesting to note an overrepresentation of SP CD8 versus SP CD4 (Fig. 2C) in AML1-ETO/GFP$^+$ thymic cells, which suggests that AML1-ETO may favor de-

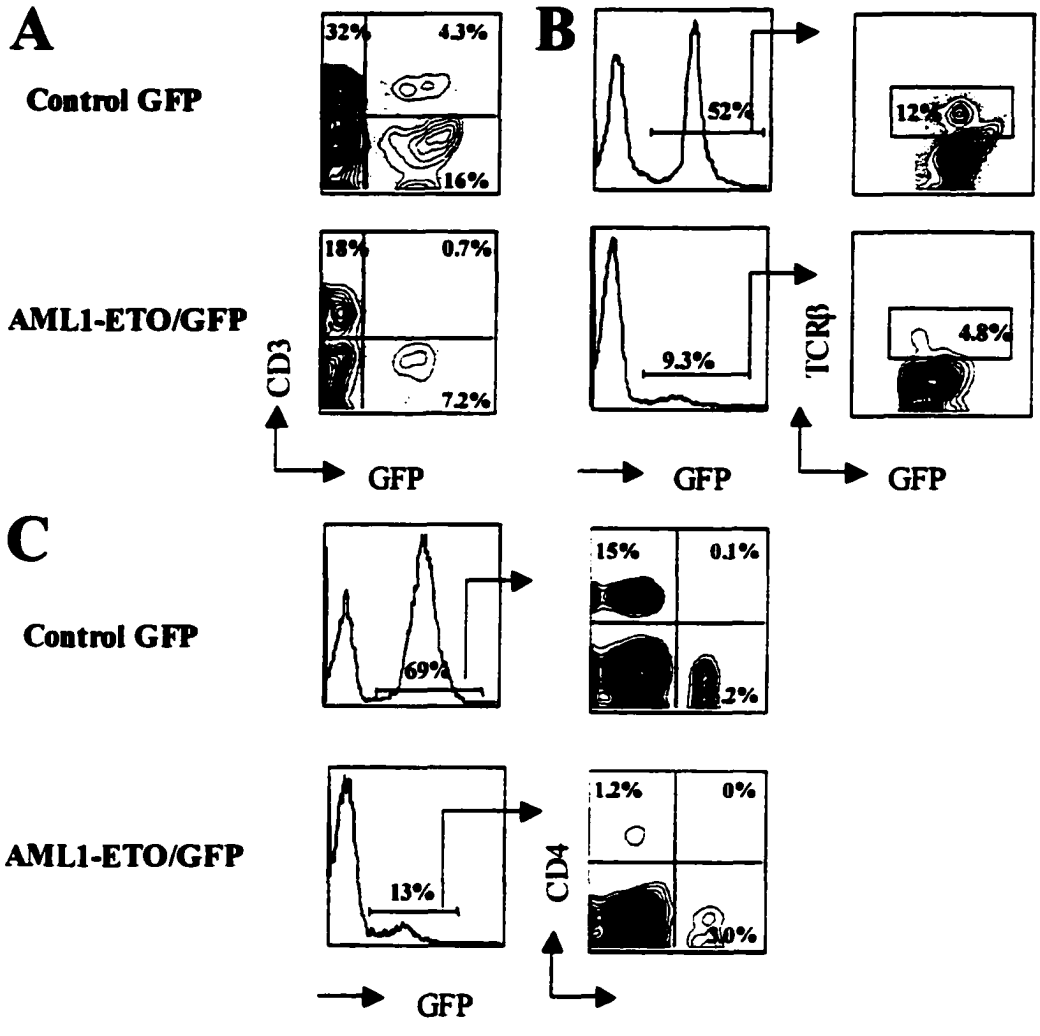

FIG. 1. AML1-ETO/GFP$^+$ peripheral blood T cells are CD3-TCRβ^{lo} and are decreased in the frequency of SP CD4 and SP CD8. Representative FACS plots of peripheral blood cells obtained from AML1-ETO and control recipients and stained with (A) T cell marker, CD3 (n = 11 [AML1-ETO]of 12 analyzed and n = 10 [control] of 11 analyzed). AML-ETO/GFP$^+$ and control GFP$^+$ cells were gated and analyzed for (B) TCRβ expression and (C) CD4 and CD8 expression (n = 4 [AML1-ETO] and n = 4 [control]).

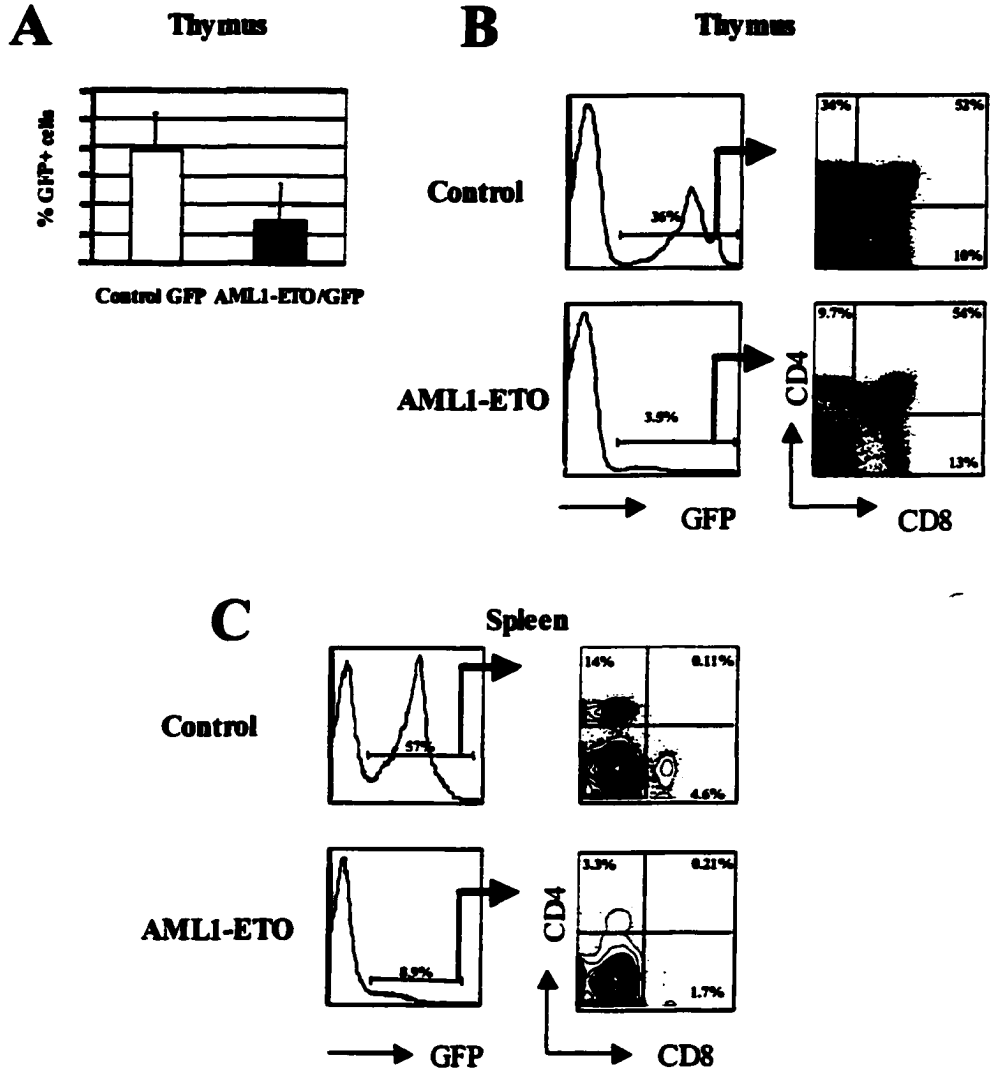

FIG. 2. Decreased frequency of SP CD4 and SP CD8 in AML1-ETO/GFP$^+$ cells in thymus and spleen. (A) Average of the percentage of control GFP$^+$ and AML1-ETO/GFP$^+$ cells in the thymi of transplanted animals (n = 9 [AML1-ETO] and n = 11 [control]). Representative FACS plots of control and AML1-ETO/GFP$^+$ gated cells stained with CD4 and CD8 in the (B) thymus and (C) spleen (n = 4 [AML1-ETO] and n = 4 [control]).

velopment of an SP CD8 cytotoxic T cell over an SP CD4 helper T cell. However, wecannot exclude the possibility that these AML1-ETO/GFP$^+$ SP CD8 cells may represent an immature CD8 cell that precedes an immature DP CD4/CD8 T cell. Analysis of the spleens of AML1-ETO recipients indicates that these AML1-ETO/GFP$^+$ SP cells migrate to the spleen.

AML1-ETO blocks early thymic precursors at the CD44$^{+/lo}$CD25$^+$ TN2 stage. In order to determine whether the low frequency of mature CD4 and CD8 T cells in AML1-ETO/GFP$^+$ cells was due to an improper development an the early T-cell precursor stage, AML1-ETO/GFP$^+$ CD3$^-$CD4$^-$CD8$^-$ (TN) cells were analyzed for CD44 and CD25 expression to delineate maturing pro-T cells undergoing TCRβ rearrangement. Flow cytometric data indicate an expansion of the AML1-ETO/GFP$^+$ TN cells at the CD44$^{+/lo}$CD25$^+$ TN2 stage (Fig. 3), which suggests an incomplete block at this T-cell stage. It is interesting to speculate that this block may be due to the inability of AML1-ETO/GFP$^+$ cells to rearrange germline TCRβ properly. Rag1–/– and Rag2–/– mice are also blocked at this stage (4).

AML1-ETO impairs mature B-cell development. We previously reported low levels of B220 (CD45R isoform) expression on AML1-ETO/GFP$^+$ peripheral blood cells compared to controls, which suggests that AML1-ETO either downregulates the B220 marker or inhibits mature B-cell development. Interestingly, Ly-5.2 (CD45R) is also expressed at lower levels on cells that express high levels of AML1-ETO (3). To address whether AML1-ETO inhibits B-cell differentiation, we analyzed

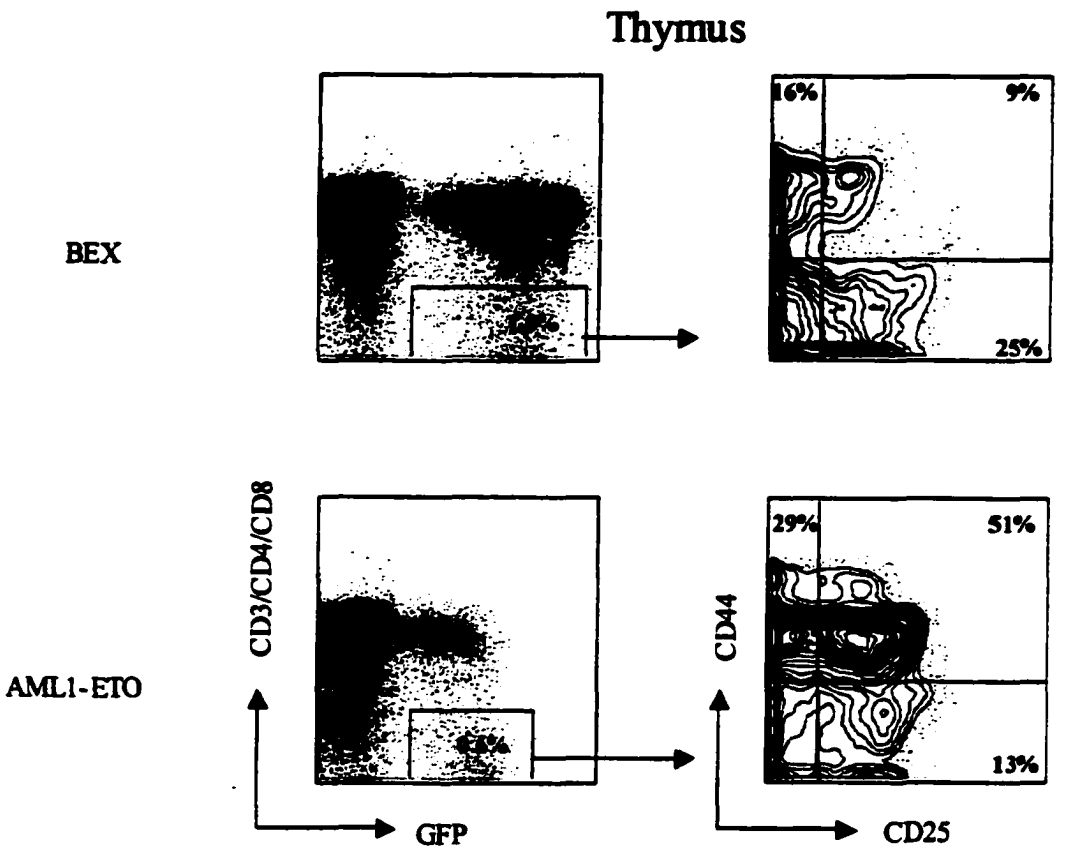

FIG. 3. Accumulation of TN AML1-ETO/GFP$^+$ cells at the CD44$^+$CD25$^+$ (TN2) stage. Representative FACS plot of control and AML1-ETO/GFP$^+$ thymic cells gated on early T-cell precursors (TN = CD3−CD4−CD8−) and analyzed for CD44 and CD25 expression Note the accumulation of CD44$^+$CD25$^+$ in the TN AML1-ETO/GFP$^+$ fraction compared to controls (n = 1 [AML1-ETO] and n = 3 [control]).

AML1-ETO recipients for mature $CD19^+$ B cells. We were unable to detect any or very few AML1- ETO/GFP^+CD19^+ B cells compared to controls (Fig. 4A). However, we detected $CD43^+$ immunoglobulin M $(IgM)^-$ (fractions A-C), $CD43^-IgM^-$ (fraction D), and $CD43^-IgM^+$ (fraction F) in the AML1-ETO/GFP^+B220^{lo} population (Fig. 4B). The greatly expanded AML1-$ETO/GFP^+CD43^+IgM^-$ population compared to controls suggests a block in B-cell development at the early pre-pro- to pro-B-cell stage.

DISCUSSION

In summary, AML1-ETO expression at the HSC level affects not only the stem cell and myeloid compartment but the lymphoid compartment, as well. AML1-ETO/ GFP^+ cells failed to give rise to mature $CD3^+$ T cells in the periphery but appeared to give rise to few SP CD4 and CD8 cells in the thymus and spleen. The decreased frequency of AML1-ETO/GFP^+ cells in the thymus further suggests that AML1-ETO appears to inhibit proper T-cell development that was confirmed by an apparent block at the TN2 ($CD44^+CD25^+$) stage.

The analysis of AML1-ETO mice also demonstrated that AML1-ETO affects B-cell development. No AML1-ETO/GFP^+ $CD19^+$ cells were observed in all AML1-ETO mice compared to controls, suggesting an early block in B-cell development. This block correlates with our observation that AML1-ETO/GFP^+B220^+ cells were mostly $CD43^+$ IgM^- $CD19^-$,which indicates an apparent block at the pre-pro-B-cell (fraction A) stage.

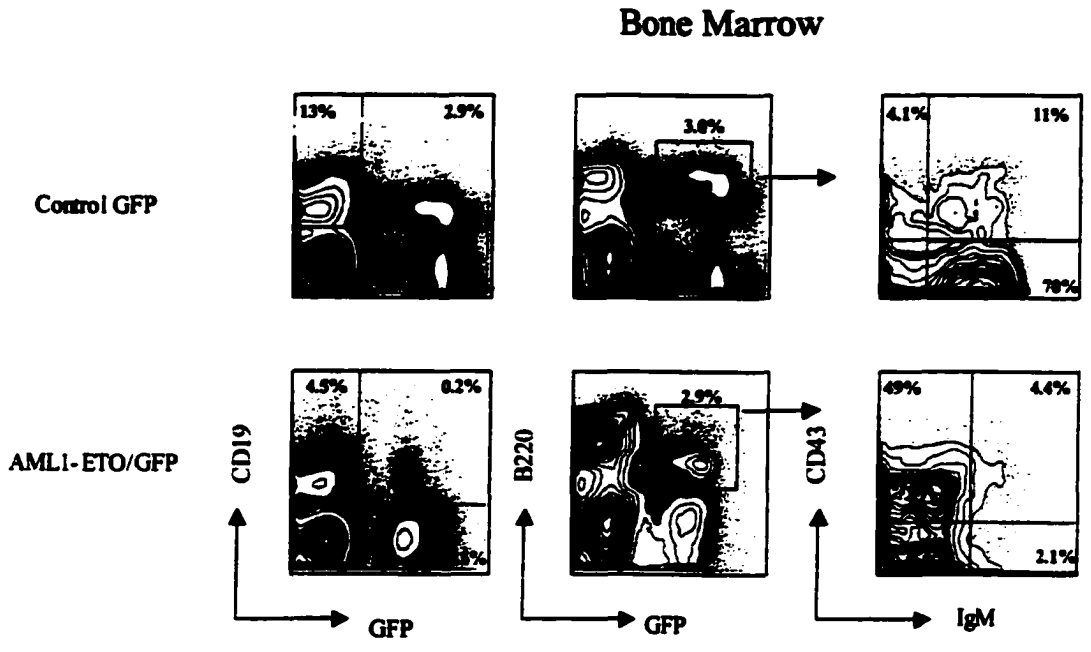

FIG. 4. Impaired B-cell development in AML1-ETO-expressing cells. Representative FACS plots of control and AML1-ETO/GFP$^+$ bone marrow cells stained for (A) CD19 expression and (B) B220, CD43, and IgM ($n = 3$ [AML1-ETO] and $n = 6$ [control]).

A role for AML1-ETO in multipotent progenitors. It is possible that the low frequency of AML1-ETO expression in the lymphoid compartment could be attributed to the partial block in AML1-ETO$^+$ HSC to differentiate (3). The fact that we observe more AML1-ETO-expressing cells in the myeloid compartment versus the lymphoid compartment suggests a skewing of the AML1-ETO$^+$ HSC toward the myeloid lineage. This skewing could be explained by an increase in common myeloid progenitor versus the common lymphoid progenitor cells. This possibility is currently being explored. Lymphoid progenitors that do arise from AML1-ETO$^+$ HSC are then blocked in their ability to give rise to mature lymphoid cells. It appears that AML1-ETO affects commitment not to the T-cell lineage but rather to the B-cell lineage because there are no AML1-ETO$^+$CD19$^+$ cells.

A role for AML1-ETO in B-cell development. The absence of AML1-ETO$^+$ CD19$^+$ cells suggests that AML1-ETO may inhibit CD19 expression at the cell surface. The gene for B-cell-specific activator protein (PAX5; also called BSAP) is a transcription factor required for B-cell commitment and regulates CD19 (18). AML1 is known to interact with PAX5 in upregulating btk expression at its promoter in Ba/F3 cells (10). It is possible that AML1-ETO may function as a dominant-negative inhibitor of PAX5 activity and thereby downregulate CD19 expression.

A role for AML1-ETO in early thymic precursors. Analysis of enhancer elements of *TCR*α, -β, -γ, and -δ reported several core-binding sites in these enhancers, suggesting a role for Runx family members in transcriptional activity of these elements

(8, 23, 25, 32). Enhancer activity is required for proper T-cell development in that deletion of the Eβ in mice led to an inhibition of V(D)J recombination at the targeted TCRβ locus and a block in αβ differentiation (9). We speculate that expression of a dominant-negative AML1-ETO abrogates enhancer activity, thereby inhibiting proper rearrangement of the TCRβ locus. However, γδ rearrangement does not appear to be affected in these mice because AML1-ETO/GFP$^+$ cells give rise to γδ T cells similar to γδ T cells of control GFP$^+$ cells (data not shown). Interestingly, conditional knock-outs of *loxP-AML1* and *cre-lck* mice have reported a block at the TN3 stage immediately after the stage where we observed a block.

A role for AML1-ETO in CD4 SP versus CD8 SP fate. It appears that a delicate balance between the levels of AML1 expression must be tightly controlled in determining the fates of CD4 SP versus CD8 SP cells. Thymocytes from transgenic AML1 mice were skewed toward CD8 SP versus CD4 SP cells, even with MHC class I absent, but not with both MHC class I and II absent (5). However, heat stable antigen staining suggested that the CD8 SP cells were not mature. Heterozygous AML1+/– mice and mice expressing a dominant-negative form of AML1 also exhibited a reduction in the number of thymocytes and a skew in CD8 SP versus CD4 SP cells (6).

Consistencies with clinical data. These studies provide clues to why patients with the t(8;21) translocation fail to give rise to AML1-ETO$^+$ T cells and few B cells and explain why these patients are immunocompromised (14). The fact that AML1-

ETO$^+$ cells undergo various blocks in development suggests a panhematopoietic role for AML1 at many stages of hematopoieisis, including the stage for HSC.

MATERIALS AND METHODS

Generation of AML1-ETO-expressing mice. Mice were generated as previously described (3).

Antibodies/FACS analysis. Single cell suspensions of bone marrow were obtained by flushing the four long bones of AML1-ETO or control GFP transplanted mice with phosphate-buffered saline. The following monoclonal antibodies from PharMingen (San Diego, CA) were used in various combinations: α-IgM-Cy5 (331), α-CD43-PE (S7), α-CD-19-bio (1D3), α-B220 (CD45R)-Cy5PE (RA3-6B2), α-CD3-PE (145-2C11), α-CD4-PE (L3T4), α-CD8-PE (53-6.1), α-CD44-Cy5PE (1M7), and α-CD25-bio (7D4). Cells were stained for 20 min at 4°C in phosphate-buffered saline/2% fetal calf serum and then washed. Streptavidin-APC and avidin-TR were used as secondary antibodies for cells stained with biotinylated antibodies. Flow cytometric analysis was performed on either a FACSCalibur (Becton Dickinson) or MoFlo flow cytometer (Cytomation, Inc.) and excluded dead cells that stained positive for propidium iodide.

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CONCLUSIONS

An HSC transformed by a genetic mutation or mutations is responsible for the establishment of a leukemic hierarchy. These genetic mutations frequently include chromosomal translocations that have been specifically associated with particular AML and ALL subtypes. Previous models have sought to determine whether the expression of the AML1-ETO fusion protein that arises from a t(8;21) translocation found in the AML FABM2 subtype is the cause for the leukemic disease (33, 46, 53, 70, 75, 99, 113, 150, 160, 162). Embryonic lethality in the knock-in models has precluded such studies. Other models, including the inducible AML1-ETO animals, have not clearly demonstrated whether expression of AML1-ETO is targeted to the HSC; however, these models have established that AML1-ETO alone is not sufficient to AML in mice. The lack of selectable markers that are coexpressed with AML1-ETO has also made it difficult to dissect the contribution of this fusion protein in hematopoietic development. Coexpression of the GFP with AML1-ETO and introduction of AML1-ETO into HSC ($Sca\text{-}1^{+}c\text{-}Kit^{+}Lin^{-}$) by retroviral transduction in our model allows us to properly investigate the effects of AML1-ETO in hematopoietic development and its contribution to a preleukemic state in our animals. In our approach, we have been able to bypass the embryonic lethality observed in knock-in models. Our findings include the detection of specific myeloid abnormalities, the presence of impaired lymphoid development, and the discovery of unique LSC properties (26).

Careful analysis of the myeloid compartment in our AML1-ETO recipients has confirmed previous observations of an increase in the number of myeloid-colony-forming progenitors. The myeloid progenitor activity was increased fourfold over that of controls at 2 months posttransplant and reached 10-fold at 10 months posttransplant, a findingconsistent with an increase in myeloblasts and promyelocytes seen in 10 months posttransplanted animals that represents up to 10% of the bone marrow. There was also a significant decrease in the number of mature eosinophils that can be associated with an increase in eosinophilic myelocytes that exhibited basophilic staining compared to controls. Northen blot analysis revealed a twofold decrease in the level of C/EBPα transcript in myeloid-sorted AML1-ETO/GFP$^+$ cells compared to controls and may explain the decrease in the number of mature neutrophils in the AML1-ETO/GFP$^+$ population. Downregulation of C/EBPα has also been observed in patients with the t(8;21) (104). Pabst et al. determined that downregulation of C/EBPα is attributed to the inhibition of C/EBPα autoregulation of its promoter through the ETO domain of AML1-ETO (104).

Studies on the association of AML1-ETO with C/EBPα have led to the speculation of a potential binding of AML1 to other CCAAT-enhancer-binding protein family members, particularly CCAAT-enhancer-binding protein epsilon (C/EBPε), which plays a role in the later stages of myeloid differentiation (60). Knock-outs in C/EBPε demonstrated the requirement of this transcription factor to produce normal neutrophils or eosinophils and the expression of secondary and tertiary granule proteins (159). C/EBPε functions downstream and is regulated by C/EBPα. It is possible that AML1-ETO may

directly inhibit C/EBPε or may indirectly do so by inhibition of C/EBPα. This possibility is currently being investigated in our laboratory.

The contribution of AML1-ETO to the lymphoid lineage in patients has not been thoroughly investigated because much of the attention has been devoted particularly to cell lines and the role of AML1-ETO in myeloid development. However, Higuchi et al. reported the lack of AML1-ETO expression in the thymus of their pI-pC-treated AML1-ETO stop/+/IFN-α/β-inducible-*Mx1-Cre* double transgenic mice upon activation of the AML1-ETO transgene. These observations have been further supported by the absence of the *AML1-ETO* transcript by RT-PCR analysis of 500 T cells from patients expressing AML1-ETO in their HSC. These observations suggest an inhibitory role of proper T-cell development from the AML-ETO-expressing cells. Our initial studies of AML1-ETO/GFP$^+$ peripheral blood cells supported this hypothesis, where we observed very few AML1-ETO/GFP$^+$/CD3$^+$ cells. Further investigation into the early T precursors suggested that the reduction in mature T cells could be attributed to the block and expansion of AML1-ETO/GFP$^+$CD3$^-$CD4$^-$CD8$^-$CD44$^+$CD25$^+$ cells, which represent pro-T cells in the process of rearranging their TCRβ locus. It is possible that AML1-ETO$^+$ cells may be inefficiently blocked at the triple negative CD44$^+$ CD25$^+$ stage as a result of their inability to rearrange the TCRβ locus properly. Rescue of these cells by a transgenic TCRβ is necessary to address whether this hypothesis holds true.

AML1-ETO expression in the HSC compartment also affected B-lineage development. No CD19$^+$ B cells were detected in the bone marrow of all AML1-ETO recipients, suggesting a block in the commitment to B-cell development. This hypothesis

was rejected upon further analysis of $B220^+$AML1-ETO/GFP^+ cells in the bone marrow and spleen that were immunoglobulin M $(IgM)^+$. It is tempting to speculate that AML1-ETO may be inhibiting PAX5 activity, which is responsible for the expression of the CD19 marker. This speculation is consistent with the finding of an increased frequency of $CD43^+IgM^-$ cells in the $B220^+$AML1-ETO/GFP^+ population that represents the early pre-pro-B to pro-B-cell precursors.

The investigation of HSC in our AML1-ETO mouse model has led to the discovery of unique properties exhibited by AML1-ETO-expressing HSC. The absolute number of HSC ($Sca\text{-}1^+c\text{-}Kit^+Lin^-$) at 2 months posttransplant was increased threefold compared to that of controls and 29-fold by 10 months posttransplant. Differences in the percentages of AML1-ETO/GFP^+ cells in the bone marrow and HSC compartment at 2 months posttransplant were large and suggest an inhibitory effect by AML1-ETO to HSC differentiation. Both sets of data can be explained by an alternative choice for the AML1-ETO^+ HSC to adopt a fate that favors self-renewal at the expense of differentiation. The increased in vitro replating potential observed in primary AML1-ETO progenitors isolated from the knock-in models supports this claim. The recruitment of HDAC and corepressors such as mSin3A and N-CoR to AML1-binding sites by the ETO domain of AML1-ETO could lead to the inaccessibility of the transcriptional complex to activate genes induced in differentiating HSC. If this process is the case, it may very well be that the adoption of self-renewal is the default pathway for an AML1-ETO HSC. Alternatively, the accumulation of HSC and the partial block in differentiation observed in AML1-ETO animals could be due to a decreased rate of cell turnover or altered cell cycle status.

We have demonstrated by serial transplantation that the presence of an AML1-ETO HSC is required for the maintenance of a preleukemic phenotype that we observe in our mice. These cells are capable of self-renewing and give rise to myeloid developmental abnormalities and impaired lymphoid development. Cytogenetic analysis of fetuses in utero and of patients previously diagnosed with a t(8;21) AML in remission have documented the presence of the AML1-ETO transcript and further support the idea that secondary hits are required for full-blown leukemia (152). Accumulation of HSC observed in our mice provides a bigger pool of cells for the accumulation of secondary hit mutations. Gain-of-function mutations such as constitutively active *c-Kit* or N-*ras* (mutated in 30 to 50% of AML cases) might cooperate with AML1-ETO to expand the myeloid developmental blocks seen in our model and therefore cause leukemia. This hypothesis is currently being tested in our laboratory.

Current therapies for AML patients rely on targeting rapidly proliferating leukemic cells by chemotherapeutic agents or more recently by disrupting constitutive molecular signaling pathways such as the receptor tyrosine kinase pathway that are shared among certain AML subtypes. Although these therapies may be successful in prolonging survival, detection of the *AML1-ETO* transcript by RT-PCR in patients in remission provides evidence that the leukemic clone still exists. With the identification of the LSC at the root of AML, it is important for clinicians and scientists to revisit therapeutic approaches engineered to target the LSC properly. Developments in immunotherapy provide a promising future for the specific "killing" of LSC. Therapies could involve the administration of a toxic agent conjugated to an antibody specific for a unique marker on the target cell. The immunoconjugate drug Mylotarg, a humanized anti-

CD33 immunoglobulin G4 antibody to the potent antitumor antibiotic calicheamicin, has recently been approved by the Food and Drug Administration against $CD33^{+}$AML in older patients who are poor candidates for aggressive reinduction regimens (4, 124, 125). Other drug immunoconjugates specific for CD33, CD45 (panhematopoietic marker except for pla-telets and red blood cells), and CD66 (present on mature cells and not leukemic blasts) that have been tested contain a radionuclide that emits α particles in an extremely short path length such as ^{213}Bi. An interesting candidate for radioimmunoconjugate therapy is the IL-3α receptor (CD123) expressed solely on the LSC and not on normal stem cells (52). Other candidate markers on LSC can be properly identified by cDNA microarrays.

Our model has brought new insights into the understanding of the t(8;21) translocation and the existence and unique properties of the LSC that could be exploited for therapeutic interventions. We hope that the methods used to generate this mouse model by retroviral transduction of HSC will be applied to studies of other chromosomal translocations, as well.

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APPENDIX A

REPRINT PERMISSION LETTER

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Article Title:

Hematopoietic Stem Cell Expansion and Distinct Myeloid Developmental Abnormalities in a Murine Model of the AML1-ETO Translocation

Coauthors

Name (type)	Signature	Date
Zheng Zhang	Zheng Zhang	8/29/2002

APPENDIX B

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Article Title:

Impaired Lymphoid Development in a Mouse Model of the AML1-ETO Translocation

Coauthors

Name (type)	Signature	Date
Rose Ko		8/29/2002

APPENDIX C

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

MEMORANDUM

DATE: November 29, 2001

TO: Christopher Klug, Ph.D.
WTI-387 3300
FAX: 934-1875

FROM: Clinton J. Grubbs, PhD, Chairman
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use COmmittee (IACUC) on November 29, 2001.

Title of Application: Analysis of a Murine Model of the AML1-ETO Translocation

Fund Source: NIH

Apn: 010405645

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
B10 Volker Hall
1717 7th Avenue South
205.934.7692 • Fax 205.934.1188
iacuc@uab.edu
www.uab.edu/iacuc

The University of
Alabama at Birmingham
Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Cristina G. de Guzman

Graduate Program Medical Genetics

Title of Dissertation Characterizing the role of the AML1-ETO Translocation in Hematopoietic Stem Cells

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name	Signature
Christopher A. Klug, **Chair**	
Andrew J. Carroll, III	
Peter D. Emanuel	
Richard D. Lopez	
J. Michael Ruppert	

Director of Graduate Program

Dean, UAB Graduate School

Date 2/5/2003