Cooperation between the partial tandem duplication of *Mll (Mll PTD)* and internal tandem duplication of *FLT3 (FLT3 ITD)* in the pathogenesis of acute myeloid leukemia (AML)

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by

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ABSTRACT

Acute myeloid leukemia (AML) with normal cytogenetics characterizes 40-50% of all acute leukemia cases [10]. Previous studies have shown that the Fms-like tyrosine kinase-3 internal tandem duplication (\textit{FLT3 ITD}) mutation is found in \textasciitilde30\% of patients with AML. Another mutation, known as the partial tandem duplication of the \textit{Mixed Lineage Leukemia} gene (\textit{MLL PTD}) is found in \textasciitilde4-7\% of AML patients with no other chromosomal disruptions. In a subgroup of patients harboring the MLL PTD, co-presence of the FLT3 ITD has also been found. Previously, we have generated and characterized the Mll PTD mouse model, and found that these mice live an essentially disease free life span with no signs of acute leukemia. Therefore, we wanted to determine whether the introduction of a second mutation such as the \textit{FLT3 ITD} could cooperate with the Mll PTD to generate leukemia in a mouse model in order to better understand the pathogenesis of this disease. Indeed we found that the combination of \textit{MLL PTD} and \textit{FLT3/ITD} induces leukemia in our mouse model between 35-65 weeks of age in 100\% of the mice analyzed thus far. Understanding how the \textit{FLT3} cooperates with the \textit{Mll PTD} in leukemic transformation will, hopefully identify novel pathways that could be used to identify new strategies to selectively target these leukemia cells in AML patients.
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INTRODUCTION

The Mixed Lineage Leukemia (MLL) gene

Recurring chromosomal translocations are characteristic of human acute leukemias and the reproduction of translocations using mouse models are being studied to generate better understanding of leukemogenesis and new therapeutic approaches. The Mixed Lineage Leukemia (MLL) gene, the human homolog of Drosophila Trithorax gene is located at 11q23 in the human genome. MLL is located in a region of the human genome which is frequently rearranged in leukemia [17]. Studies suggest that normal MLL is required for hematopoietic stem cell (HSC) development or the differentiation of the multipotent HSC to lineage restricted hematopoietic progenitor cells (HPC) [3]. Translocations found in acute leukemia involving 11q23 and result from the fusion of MLL with more than 40 possible different non-homologous partner genes. In each case, the MLL protein retains its AT hook and methyltransferase domain at N-terminal end while losing the SET domain in its C-terminal portion.

Although most of these gene rearrangements involving MLL result in chromosomal translocations, MLL itself was also found to be rearranged in 4-7% (of 956 cases) of patients with acute myeloid leukemia (AML) and normal cytogenetics [1, 11, 15]. This rearrangement consists of a partial tandem duplication of the N-terminus of MLL, in which a defined segment of the N-terminus is duplicated and inserted in tandem with an otherwise full length MLL (Fig. 1).
To create a mouse model of the *Mll* PTD exons 5-11 or 5-12 (formerly designated exons 2-6 & 2-8) were inserted into intron 4 of the endogenous *Mll* gene.

Understanding the underlying molecular mechanisms involving *Mll* PTD-mediated leukemogenesis is especially important in patients harboring the *MLL* PTD, which consistently have shorter disease-free survival rates [15]. It is our hope that by generating the mouse model of the *Mll* PTD, we will begin to understand how the *Mll* PTD actually contributes to the leukemic phenotype and can then generated more appropriate treatments for this subgroup of patients.

*The FMS-Like Tyrosine kinase 3 (FLT3) gene.*

Another gene aberration that appears frequently in acute leukemia involves mutations of the FMS-like tyrosine kinase 3 (*FLT3*) gene. *FLT3* encodes a class III receptor tyrosine kinase [12] and is important for normal hematopoiesis [5, 16]. The *FLT3* gene is structurally related to c-kit (KIT), c-fms (FMS), and platelet derived growth factor (PDGF) receptors, which are members of the class III receptor tyrosine kinase [12]. Normal *FLT3* activates upon the binding of *FLT3* ligand to the *FLT3* receptor, which induces intracellular signaling pathways essential to regulation of cell proliferation, apoptosis, maturation of myeloid and lymphoid progenitor cells, dendritic cells, and natural killer cells [2].

Somatic mutations involving the *FLT3* gene include internal tandem duplication (ITD) of the juxtamembrane domain or point mutations. *FLT3* mutations that have been identified in 7-9% of AML patients appear to activate the tyrosine kinase domain (KD) through receptor dimerization in a *FLT3* ligand-independent manner [12]. A number of studies have shown that *FLT3* ITD mutations are of significant clinical consequence and
may be associated with disease progression [6] and an increased risk of relapse and shorter overall survival [4]. Previous studies have found that FLT3 ITD mutations occur in approximately 17-34% of adult AML, 5-17% pediatric AML, 3% in MDS, and 3% in pediatric ALL [16].

Two classifications of FLT3 mutations have recently been described, those which interfere with the regulatory function of the juxtamembrane region which is seen with internal tandem duplication of FLT3 (ITD) and those involving KD point mutations which occur within the activation loop (AL) of the second kinase domain [16] (Fig. 2).

Both mutations constitutively activate the receptor’s tyrosine kinase activity in the absence of the ligand. The FLT3 ITD have been shown to downregulate a number of important transcription factors, CEBPα & Pu.1, that are involved in normal myeloid proliferation and differentiation [12]. In ITD mutations, FLT3 ITD receptors dimerize in ligand-independent manner, leading to autophosphorylation of receptor through constituitive activation of the tyrosine kinase domain [12]. FLT3 ITD expression was also shown to suppress radiation-induced apoptosis [12].
Collaboration between the MLL PTD and FLT3 ITD in leukemogenesis

In unselected cases of AML, a higher incidence of MLL PTD in FLT3 ITD positive patients was significantly higher than in FLT3 ITD negative patients [13]. The coexistence of both genetic aberrations may suggest either a common or cooperating underlying mechanism of transformation. To investigate further address this hypothesis as well as better understand the role of the Mll PTD in generating leukemia, members of the Caligiuri Lab developed a mouse ‘knock-in’ model of the Mll PTD. These mice express the Mll PTD transcript as well as the concomitant Mll wildtype (WT) transcript. Although the Caligiuri lab observed aberrant proliferation in the hematopoietic progenitor cells (HPCs) in the Mll^{PTD/wt} mouse, these mice do not develop leukemia during their normal lifespan (>2 years) (Table 1). Through colony forming assays (CFU), competitive repopulation methods, and assessment via bromodeoxyuridine (BrdU), the lab’s findings suggest that the role of Mll PTD is to ready the hematopoietic compartment to be further induced into a leukemic phenotype when in the presence of a second hit in differentiation and/or apoptosis.

Recent studies show that FLT3 is overexpressed in AML with MLL translocations. In human AML, the MLL PTD has been shown to simultaneously occur with tyrosine kinase mutations, such as the FLT3 internal tandem duplication (ITD) [13, 15]. Internal tandem duplications (ITD) within the juxtamembrane domain of FLT3 in patients with AML occur in approximately 25% (119/523) of patients with normal cytogenetics [4]. To further investigate how the FLT3 ITD cooperates with the MLL PTD in leukemic transformation, we obtained mice heterogenous for the FLT3 ITD from our collaborators at the Gilliland Lab. Dr. Gilliland’s group has shown that the Flt3^{ITD/ITD} and Flt3^{ITD/wt}
mice breed normally and live a relatively normal life span. However, they present with enlarged livers, increases in their hemoglobin and platelet counts, splenomegaly, and increases in mature monocyte and granulocyte populations. Furthermore, these knock-in mice display a block in B cell development characterized by increases in B220⁺CD43⁻ pre-B cells and decreases in the more mature B220⁺IgM⁺ cell types. *Flt3*<sup>ITD/ITD</sup> and *Flt3*<sup>ITD/wt</sup> mice also have increases in the number of hematopoietic progenitor cells (HPCs) as quantified by colony forming unit (CFU) assays. However, unlike the *Mll*<sup>PTD/wt</sup> progenitors, these progenitor cells do not display concomitant increases in self-renewal, as determined by serial re-plating assays [7]. Two groups who have generated knock-in models of *FLT3* ITD confirm this observation and although these mice display myeloid-associated phenotypes, fatal leukemia was not detected over the normal lifespan of these mice [7, 9].

Although both *Flt3* ITD and *Mll* PTD mice both display aberrant hematopoietic development, a critical step in the leukemogenic pathway, neither develop leukemia over their normal lifespan. However, *Mll* PTD mice and *Flt3* ITD mice do not acquire acute leukemia separately. These studies support the Knudson’s two-hit hypothesis in that these mutations alone are not sufficient to generate leukemia by themselves, and the presence of a ‘second-hit’ is needed in order to generate full-blown leukemia. The purpose of this study is to determine if the presence of both the *MLL* PTD and *FLT3* ITD mutations are sufficient for leukemogenesis and to use this novel leukemia model to investigate novel therapies not only for patients harboring both mutations, but possibly patients harboring only one of these mutations.
RESULTS

To investigate whether the FLT3 ITD mutation cooperates with the MLL PTD and thus providing the “second hit” in for cellular transformation, Flt3ITD/wt and MllPTD/wt mice were mated to create and characterize the MllPTD/wt Flt3ITD/wt double mutant mouse model. Flt3ITD/wt pups were mated each generation to pure MllPTD/wt -C57Bl/6J mice to generate a ‘pure’ MllPTD/wt Flt3ITD/wt –C57Bl/6J line. The genotype of the MllPTD/wt Flt3ITD/wt mice was determined via PCR analysis (Fig. 3). We have currently backcrossed these mice 8 generations. MllPTD/wt Flt3ITD/wt mice are born at normal Mendelian ratios with no signs of delayed growth. After initial matings, we observed that all of the MllPTD/wt Flt3ITD/wt mice died within 40 weeks of age while none of the littermate controls showed any premature death. Subsequent generations of these mice have lived up to 65 weeks of age, but still died prematurely below the expected natural lifespan (>104 weeks). In fact, no MllPTD/WT Flt3ITD/WT mice yet to survive past 65 weeks of age (n>15 mice, p<0.001). However, all MllPTD/wt and Flt3ITD/wt littermate controls all live normal life spans.

Mice were monitored weekly for loss of activity and/or weight, but in early litters, mice showed no visible signs of disease, making it exceedingly difficult to catch the MllPTD/WT Flt3ITD/WT mice before death. In subsequent litters, the animals were observed daily while complete blood counts (CBCs) and weights were taken weekly. MllPTD/WT Flt3ITD/WT mice displaying signs of disease (as assessed by CBCs or loss of activity) were sacrificed, and tissues were harvested to determine cause of death. At time of death, mice(n=4) presented with anemia and with a greater than 20 fold-increase in WBCs compared to littermate controls (Fig. 4). More importantly, cytospins of the bone
marrow and spleen, and corresponding blood smears all showed 20-80% of blasts present. Furthermore, immuno-histochemistry (IHC) revealed infiltration of leukemic blasts in all tissues examined to date (Fig. 5).

In two of the $Mll^{PTD/WT}$ $Flt3^{ITD/WT}$ mice, the leukemia lineage was determined using flow cytometry (Fig. 6). These analyses revealed that both mice were full of leukemic blasts by morphology but differed in their immunophenotype. One mouse, identified as PF-59, had a markedly expanded Mac1+/Gr1+ myeloid population (~53%) in the spleen, with relatively little CD3 positive T cells or B220 positive B cells. In examining PF-59’s tissues, it was evident that there was some limited degree of myeloid maturation occurring among the predominance of immature blast forms. The other mouse, identified as PF-72, showed no significant Mac1+/Gr1+ double positive population but rather an increased B220 (dim positive) population and no significant CD3 T cell population. Unlike mouse PF-59, mouse PF-72’s also showed a significant c-kit positive population (~35%).

The immuno-histochemistry results from PF-59 for myeloperoxidase (MPO) showed a significant degree of expression in a large proportion of these blasts (Fig. 7). Interestingly, there was also a significant subset that appeared positive for Terminal Deoxynucleotidyl Transferase (TdT) as well as a subset that appeared to show variable, weak B220 expression. In mouse PF-72, the initial flow results which showed a significant B220 positive population were confirmed by IHC for B220 in showing a significant staining pattern for B220, with a subset staining positive for TdT, but no CD3 staining. Interestingly, there was some significant staining for a small proportion of the blasts for MPO, which was consistent with the dim Mac1 staining seen initially by flow.
DISCUSSION

AML is a heterogeneous disease and can result from unique gene fusions as a result of chromosomal translocations but can also develop in cells in which no other cytogenetic alterations are visible (i.e. normal cytogenetics). Some of these intra-gene disruptions can lead to incorrect activation of signal transduction pathways, alterations of growth factor receptors, and ultimately disease [10]. Our studies on a double knock-in mouse model demonstrates that FLT3 ITD can collaborate with the MLL PTD to induce acute leukemia in an in vivo mouse model. 100% of the MllPTD/WT Flt3ITD/WT mice observed thus far, die between 35-65 weeks of age with massive leukemic infiltration of their organs. Consequently, our studies suggest that cooperation between different mutations such as the MLL PTD and mutants leading to constitutive FLT3 activation, ensures full transformation of hematopoietic cells and that the combination of these mutations are interdependent in the pathogenesis of AML.

As previously mentioned, our MllPTD/WT Flt3ITD/WT mice were observed to have infiltration of disease in the hematopoietic organs. In fact, up to ~24 hours before death, these mice display normal activity, demonstrating the acute nature of the disease. The immunohistochemistry studies revealed that two MllPTD/WT Flt3ITD/WT mice had acute leukemias with biphenotypic features. In the two cases, Mouse PF-59’s blasts show a greater degree of MPO expression while Mouse PF-72’s blasts expressed a greater degree of B220 staining pattern. FLT3 is known to be overexpressed at the RNA and protein level in most B lineage and AML [12]. As a result, while Mouse PF-59 appears to display a more myeloid-predominant phenotype, Mouse PF-72 appears to display a more lymphoid-predominant phenotype.
Understanding how the *MLL* PTD may contribute to leukemogenesis in combination with the *FLT3* ITD is very important. With our unique mouse model expressing both the *Mll* PTD and *Flt3* ITD, we can begin to assess the contribution of the *MLL* PTD and *FLT3* ITD before and after leukemic transformation and positively impact AML patients harboring these mutations. Further characterization of this leukemia which includes examining altered epigenetic modifications at the promoters of *MLL* downstream target genes may lead to innovative therapeutic approaches. Furthermore, *FLT3* selective inhibitors may be used in combination with agents that target disruptions caused by the *MLL* PTD.

Because mutant forms of *FLT3* are energetically difficult to fold, they appear more dependent on chaperone proteins [12]. One way to target mutant *FLT3* would be to use inhibitors of chaperone proteins like Hsp90 [12]. Another way to inhibit *FLT3* would be by using anti-*FLT3* antibodies to block binding to FL (*FLT3* ligand). Already, some *FLT3* inhibitors have been tried in clinical trial in relapsed or refractory AML patients, where some or all had *FLT3* mutations [12]. *FLT3* inhibitors can work to suppress *FLT3* tyrosine kinase activity and induce growth arrest/apoptosis in leukemia cells expressing *FLT3* mutants [16]. However, *FLT3* mutations occurring in the setting of acute leukemia is but one of several somatic genetic alterations required to fully transform cells and may need combination therapy to be fully effective.

Our model will help explore the role of these two leukemogenic mutations in the development of AML and we hope to identify new aberrant pathways that may subsequently lead to novel targeted therapies and new treatments for AML patients with these mutations.
MATERIALS AND METHODS

*Mice and genotype analysis*

Mice heterozygous for the *FLT3* ITD were obtained from our collaborator, Dr. D. Gary Gilliland at Harvard University. Mice harboring the *FLT3* ITD at the endogenous *FLT3* locus were maintained on an FVB background. Mice heterozygous for *MLL* PTD were maintained on a pure C57B1/6J background and were crossed with the *FLT3*\(^{WT/ITD}\) mice. In each generation *FLT3*\(^{WT/ITD}\) offspring were bred to pure *Mll*\(^{PTD/WT}\) - C57Bl/6J to generate the *Mll*\(^{PTD/WT}\) *FLT3*\(^{WT/ITD}\) double mutants on a pure C57Bl/6J background.

Genotyping was performed via PCR analysis. DNA samples from the tail were prepped using the Qiagen DNeasy DNA extraction kit. Both the *FLT3*\(^{ITD/WT}\) and the wildtype *FLT3*\(^{WT/WT}\) alleles were detected at ~200 bp and ~220 bp respectively using the following primers Flt314F (5’-AGG TAC GAG AGT CAG CTG CAG ATG-3’) and Flt314R (5’-TGT AAA GAT GGA GTA AGT GCG GGT-3’) and the following parameters: 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 50s (see fig. 3). The *Mll*\(^{PTD/WT}\) mice were genotyped by PCR with primers 642F (5’–GAG CCT TGG CCC GAA TGA AAC TGT-3’) and 643R (5’–CCG GCG AAC GTG GCG AGA AA-3’), and the PTD mutant allele was detected at ~900 bp using the following parameters: 95°C for 2 min, followed by 35 cycles of 95°C for 30s, 66°C for 30s, and 72°C for 2 min, and 72°C for 10 min.
Complete Blood Count (CBC) test

Mice starting at 16 weeks of age were bled weekly using submandibular bleeding methods. Collected blood samples were submitted to the Veterinary Teaching Hospital at The Ohio State University, and the reported white blood counts (WBCs) were compared to littermate controls, weekly to monitor the progression of disease in the mice.

Histopathology

Harvested tissues at the time of death were fixed for histological examination in 10% formalin and sent to Goss Labs and the Veterinary Teaching Hospital at The Ohio State University.

Peripheral blood smears and bone marrow and spleen cytospins were prepared with Wright-Giemsa staining for morphologic evaluation.

Flow Cytometry

For multiparameter flow cytometry, bone marrow, spleen, and peripheral blood cells from \( Mll^{PTD\text{WT}} \ Flt3^{ITD\text{WT}} \) mice PF-59 and PF-72 were analyzed via standard protocols for flow cytometry using antibodies Gr1, Mac1, c-Kit, B220, and CD3. Immunofluorescent analysis of antigen expression was performed as previously described [7, 8]

Immunohistochemistry (IHC)

Immunohistochemistry was performed on \( Mll^{PTD\text{WT}} \ Flt3^{ITD\text{WT}} \) mice, PF-59 and PF-72, cell blasts via standard protocols using primary antibodies to B220 (PharMingen), myeloperoxidase (MPO; Dako), Terminal Deoxynucleotidyl Transferase (TdT), CD3 (Dako), and Mac1 (AbCam) [7, 8].
### Table 1. Summary of phenotypic & molecular findings thus far in Mll PTD knock-in mice.

<table>
<thead>
<tr>
<th>Mll Genotype</th>
<th>Viable</th>
<th>Tissue</th>
<th>Cytokine response</th>
<th>CFU</th>
<th>BrdU Proliferation</th>
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<td>Mll&lt;sup&gt;mmt&lt;/sup&gt;</td>
<td>yes</td>
<td>BM</td>
<td>Basal</td>
<td>Basal</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
<td>Basal</td>
<td>Basal</td>
<td>Basal</td>
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<tr>
<td>Mll&lt;sup&gt;PTD/wt&lt;/sup&gt;</td>
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<td>BM or Spleen</td>
<td>increased</td>
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<td>↑</td>
</tr>
<tr>
<td>Mll&lt;sup&gt;PTD/wt&lt;/sup&gt;</td>
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<td>-</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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**Fig. 3 PCR Analysis of $Mll^\text{PTD/wt}$ and $Flt3^\text{ITD/wt}$**

A.) Sequence of PCR product for $Mll^\text{PTD/wt}$. The 1 kb+ ladder was used to determine amplification size. B.) Sequence of PCR product for $Flt3^\text{ITD/wt}$.  

Fig. 4 WBCs at time of death.
WBCs at time of death show a greater than 20 fold increase in the number of WBCs in the $\text{Mll}^{\text{PTD/wt}} \text{Flt3}^{\text{ITD/wt}}$ mice compared to littermate controls.
Fig. 5 Extensive infiltration of \( Mll^{PTD/wt} \) \( Flt3^{ITD/wt} \) leukemic cells. 

A.) BM shown here at 40X demonstrates how the disease cells have completely infiltrated and taken over the BM space. 

B.) The adrenal gland shown here at 4X demonstrates large masses of diseased cells.
Fig. 6 Lineage determination of two $Mll^{PTD/WT} Fli3^{ITD/WT}$ mice who succumbed to leukemia. Mouse PF-59 (left) developed AML (Gr1$^+$ Mac1$^+$ Kit$^{dim}$ B220$^-$ CD3$^-$) while mouse PF-72 (right) developed acute undifferentiated leukemia (Gr1$^-$ Mac1$^-$ Kit$^+$ B220$^{dim}$ CD3$^-$). Admixed among the blast population are a smaller population of more mature myeloid forms (yellow arrows), indicating that there is some limited myeloid maturation occurring.
Fig. 7 Immunohistochemistry analysis of two Mll prwrt Flt3ITD mice who developed leukemia. A.) Spleen cells of mouse PF-59 (left) and PF-72 (right). B.) Mouse PF-59 shows a lot more cells stained with MPO or with Mac1+/Gr1+ expression than PF-72. C.) Both mice show significant staining for TdT. D.) Mouse PF-72 shows significantly more staining for B220 expression. E.) Both mice show no CD3 expression.
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REFERENCES

