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Amplification of mixed lineage leukemia gene perturbs hematopoiesis and cooperates with partial tandem duplication to induce acute myeloid leukemia

Genetic alternation of the mixed lineage leukemia (MLL) gene can be found in up to 10% of acute myeloid leukemia (AML).\(^1,2\) Similar to MLL fusions and MLL partial tandem duplication (PTD), MLL amplification (MLL(n)) is reported in approximately 1% AML and myelodysplastic syndrome (MDS) associates with adverse treatment outcomes.\(^3,4\) In spite of the success in modeling AML induced by MLL fusions,\(^1\) there has been no in vivo disease model for MLL(n) leukemia. On the other hand, while previous studies have proposed a recessive gain-of-function by MLL-PTD in suppressing the expression of wild-type MLL in normal karyotype patients, no appropriate disease model has been established.

Figure 1. MLL amplification perturbs myeloid development in mouse models. Total cell counts in (A) bone marrow (2 femurs), (B) thymus and (C) spleen of the control MLL(0), MLL(10-15), MLL(80-100) and Mll(PTD/WT) mice. (D) Total colony numbers generated from bone marrow cells of MLL(n) and Mll(PTD/WT) mice. Bone marrow colonies were classified into burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-granulocyte/macrophage (CFU-GM), and CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM). (E) Absolute number and (F) relative proportion of each colony type from the bone marrow of indicated genotypes. (G) Total colony numbers formed from splenocytes of MLL(0) control, MLL(n) and Mll(PTD/WT) mice. (H) Absolute number and (I) relative proportion of each colony type from the spleen of indicated genotypes. (J) Percentages of myeloid cell populations (Total: Gr-1+ or Mac-1+, Gr-1+Mac-1+, Gr-1+Mac-1- and Gr-1-Mac-1+) in spleens of MLL(0) control, MLL(n) and Mll(PTD/WT) mice. (K) Representative flow cytometry plots showing percentages of Gr-1+Mac-1+ cell population in spleens of MLL(0) control, MLL(n) and Mll(PTD/WT) mice. (L) Splenocytes (20x10^4 cells) from different mice were seeded into methylcellulose for replating assays. Enumeration of colonies and replating of cells to the next round were performed after 6 days of culture. (M) Flow cytometry analysis of the third round of replating Mll(PTD/WT) splenic colonies. Red dots, unstained cells; blue dots, cells stained with indicated antibodies. Results in panel A to C were obtained from 5 independent experiments, panel D to I were obtained from 6 independent experiments, whereas panel J and L were obtained from 5 independent experiments. Bar graphs show mean+S.E.M. (*P<0.05, **P<0.01 and ***P<0.001, paired t-test).
AML, both Mll-PTD/WT and Mll-PTD- mice failed to develop leukemia, thus questioning the role of MLL-PTD in acute leukemogenesis and indicating a requirement of cooperating mutation(s) for full malignant transformation. Intriguingly, MLL-PTD can be found to co-exist with MLL(n) in AML patients, suggesting an alternative pathological relationship between these 2 MLL aberrations in myeloid leukemogenesis.

To gain insights into the role of MLL(n) and MLL-PTD in hematopoietic development, we generated an in vivo mouse model of MLL amplification where the full-length human MLL cDNA was expressed under the murine Scl-promoter/3’enhancer (Online Supplementary Figure S1A), which has been successfully used to drive oncogene expression in hematopoietic stem and progenitor cells. 

Figure 2. Potential cooperation between MLL amplification and MLL-PTD results in leukemic transformation in MLL(40-50)PTD/WT mice. (A) Survival curves of MLL(40-50) (n=14), Mll-PTD/WT (n=12) and MLL(40-50)PTD/WT (n=12) mice. Three out of 12 MLL(40-50)PTD/WT mice developed AML but none of the control mice showed any sign of disease (*P<0.05). (B) Representative images of spleens from MLL(0), MLL(40-50), Mll-PTD/WT and leukemic MLL(40-50)PTD/WT mice. All control mice were age-matched to leukemic mice. Scale bar, 1 cm. Total cell counts in (C) bone marrow (2 femurs) and (D) spleens of MLL(0), MLL(80-100), Mll-PTD/WT and leukemic MLL(40-50)PTD/WT mice. (E) Peripheral blood smears and cytospins of the cells prepared from the bone marrow, spleen and liver of MLL(0), MLL(40-50), Mll-PTD/WT and leukemic MLL(40-50)PTD/WT mice are shown. Leukemic blasts are indicated by blue arrows. Cells were stained with May-Grunwald-Giemsa. Scale bars represent 30 μm. (F) Relative expression levels of Hoxa9 in MLL(n), Mll-PTD/WT, leukemic MLL(40-50)PTD/WT bone marrow and MLL-AF9 leukemic cells compared to E2A-PBX leukemic cells by q-RT-PCR. (G) Relative expression levels of Hoxa9 in MLL(n), Mll-PTD/WT, leukemic MLL(40-50)PTD/WT splenocytes and MLL-AF9 leukemic cells compared to E2A-PBX leukemic cells by q-RT-PCR. (H) Relative expression of HOXA9 in human MLL(n)/PTD bone marrow cells and in normal karyotype AML (NK-AML) and MLL-AF6 expressing human bone marrow cells. Bar graphs show mean±S.E.M. (*P<0.05, **P<0.01 and ***P<0.001, paired t-test).
cells (HSPCs) resulting in AML. Two different founders with 10-15 and 40-50 copies of concatemeric transgenes were selected for establishment of stable lines containing 10-15 (MLL(10-15)) or 80-100 copies (MLL(80-100)) of MLL, respectively (Online Supplementary Methods and Online Supplementary Figure S1B,C). Western blotting and quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed the transgene expression as being, approximately, a 3.5- and 6-10-fold increase in the bone marrow and spleen of MLL(10-15) and MLL(80-100) mice, respectively (Online Supplementary Figure S2A,B). These findings are consistent with previously reported ScI-enhancer’s property and in line with the expression level of MLL in human MDS/AML patients carrying 5-10 or more than 10 copies of MLL with, approximately, a 1.5- and 9-fold increase, respectively.

To investigate the impacts of MLL amplification on normal hematopoiesis, MLL(n) mice at three months old were analyzed in parallel with MLLTDWT mice and non-transgenic control (MLL(0)). Although no significant difference in total cellularity was found in bone marrow and thymus, a significant increase in splenic cells was observed in MLLTDWT mice (Figure 1A-C). In contrast, a reduction in splenic cells was found in MLL(n) mice, especially in MLL(80-100) mice (Figure 1C), suggestive of a mild differentiation and/or proliferation defect. Consistently, the splenocytes of MLL(10-15) and MLL(80-100) mice displayed a trend towards a significant increase in the percentage of Annexin-VPF- apoptotic cells compared to those of MLL(0) mice (Online Supplementary Figure S2C), providing a potential explanation for the lower cellularity observed in the spleen of MLL(n) mice. Functionally, MLLTDWT bone marrow produced approximately 4 times more colonies than those by MLL(n) and the control (Figure 1D,E). While the ratio between each colony type remained constant in MLLTDWT, MLL(80-100) had a significant increase in the percentage of colony forming units-granulocyte/macrophage (CFU-GM) and a decrease in the percentage of colony forming unit-granulocyte (CFU-G; Figure 1F), consistent with a mild shift to immature myeloid. MLL(n) splenocytes showed a slightly but significantly higher colony forming ability (Figure 1G,H), where they consistently exhibited an increase in immature colony forming units-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) and burst forming units-erythroid (BFU-E; Figure 11). The degrees of expansion were even more notable in MLL(n) mice with a higher copy number of the MLL transgene (Figure 1G-I), suggesting an aberrant selection of proliferative myeloid clones. While there was no apparent difference in the proportions of B cells and T cells in these mice (Online Supplementary Figure S3A), an expansion of the Gr-1+Mac-1+ cell population was found in the MLL(80-100) spleen (Figure 1J,K). Consistently, a transient enhanced replateing ability was observed in MLL(n) and MLLTDWT splenocytes (Figure 1L). Compared to non-transgenic controls, MLL(10-15) and MLL(80-100) splenocytes, the latter in particular, were able to form a substantial number of secondary colonies (Figure 1L). MLLTDWT splenocytes were able to generate compact myeloid colonies even in the third round of replating (Figure 1LM). In contrast, there was no obvious difference in the replating ability of bone marrow cells (Online Supplementary Figure S3B), consistent with the observed phenotypes in these mice (Figure 1A-C).

Apart from a significant decrease in the percentage of common lymphoid progenitors (CLPs) in the bone marrow of MLLTDWT mice, further investigation into the effect of MLL(n) and PTD on early HSPC populations revealed no significant difference in hematopoietic stem cells (long-term (LT)-HSCs, short-term (ST)-HSCs), early myeloid/lymphoid progenitor (multipotent progenitors [MPPs], common myeloid progenitors [CMPs], granulocyte-monocyte progenitors [GMPs], megakaryocyte-erythroid progenitors [MEPs]), or lymphoid (B-[B220], T-[CD4+CD8] and CD4+CD8+) cell populations in their bone marrows (Online Supplementary Figure S3C-E). RT-PCR confirmed the over-expression of MLL in c-kit+ HSPC bone marrow cells, albeit to a lesser extent than in whole bone marrow cells in MLL(n) mice (Online Supplementary Figure S3F,G), suggesting that mild over-expression of MLL or MLL-PTD alone has a rather limited impact on HSPC compartments. Consistently, none of the animals developed leukemia during two years of observation (Online Supplementary Figure S3H), indicating that additional events are required for overt leukemia as observed in humans.

On the other hand, the recurrent reports of AML possessing both MLL(n) and MLL-PTD raise the intriguing possibility that these 2 different MLL aberrations may collaborate in acute leukemogenesis. To address this long outstanding question, we crossed MLL(80-100) with MLLTDWT mice to produce a novel double mutant MLL(40-50)TDWT mouse (Online Supplementary Figure S1C and S4). In contrast to MLL(40-50) and MLLTDWT controls, which did not develop any malignancy, 3 out of 12 MLL(40-50)TDWT mice succumbed to AML with immature myeloblasts and splenomegaly after 7 to 21 months (P=0.0434) (Figure 2A-D). Examination of peripheral blood revealed anemia and circulating myeloblasts (Figure 2E), which also infiltrated the spleen and liver and resulted in an alteration in their normal architecture (Online Supplementary Figure S5 and Online Supplementary Table S1). Flow cytometric analysis further confirmed expansion of Gr-1+Mac-1+ c-kitlow myeloblasts (Online Supplementary Figure S6A), which was in contrast to a drastic reduction of B220+ cells (Online Supplementary Figure S6B). These results indicate that MLL amplification and MLL-PTD cooperate to block normal myeloid differentiation and to induce their aberrant expansion leading to overt AML.

To gain further molecular insights underlying these cooperating MLL aberrations, we assessed the transcriptional and epigenetic status of HOXA9, a key downstream target for MLL leukemogenesis. By comparison with MLL(40-50), MLLTDWT controls and E2A-PBX mouse leukemic cells, which expressed basal level of HOXA9, MLL(40-50)TDWT leukemic cells, in particular those from the spleen, expressed significant levels of HOXA9; almost 10-fold higher than MLL(40-50) and twice more than MLLTDWT; showing the possibility of functional cooperation in MLL aberrations (Figure 2F-G). In addition to HOXA9, a considerably higher level of Meis1, another target for MLL leukemogenesis, was also detected in MLL(80-100) and MLL(40-50)TDWT bone marrow cells, which provides an explanation for the transformation in the double mutants (Online Supplementary Figure S7A). To further confirm this finding in the relevant human disease, we also examined the expression and epigenetic status of HOXA9 in primary human AML blasts carrying both MLL(n) and MLL-PTD (Online Supplementary Figure S7B). HOXA9 was significantly over-expressed in all 3 MLL(n)-PTD samples and MLL-AF6 leukemia as compared to normal karyotype AML (Figure 2H).
At the epigenetic level, similar to MLL-AF9 mouse leukemic cells, we observed an upregulation of the H3K4me3 and H3K79me2 activation marks and a reduction of H3K9me2 repressive mark in MLL(40-50)PTD/WT bone marrow cells, where higher levels of MLL expression were detected in comparison with non-transgenic controls or Mll-PTD (Figure 3A,B). Interestingly, Kühn et al. have observed profound enrichment of H3K79me2 marks at the highly expressed HOXA cluster locus in MLL-PTD positive leukemia cell lines. They found that DOT1L inhibition abrogated H3K79me2 and led to downregulation of MLL targets in MLL-PTD leukemia cell lines, suggesting that DOT1L is a potential therapeutic target for MLL-PTD leukemia. Consistent with the mouse model, we also observed similar histone modification patterns in human MLL(n)-PTD blasts (Figure 3C). While MLL(n)-PTD samples exhibited an increased H3K79me2 mark, it was significantly lower than that seen in MLL fusion leukemia. Conversely, H3K4me3 was invariably present at a high level in all MLL(n)-PTD samples (Figure 3C), which is in line with the detection of elevated expression of wild-type MLL in primary AML patients (Figure 3D), suggesting that H3K4me3 mediated by the MLL/SET family may also play a critical role for activation of downstream targets in these leukemias. To further test this hypothesis, pharmacological inhibition of MLL catalytic activity by a small molecule inhibitor WDR-0103 significantly reduced cell viability (Figure 3E), induced cell differentiation (Figure 3F) and repressed ribonucleic acid (RNA) expression of Meis1 (Figure 3G) of...
MLL \((40-50)^{\text{PTD}}\) transformed cells, supporting the fact that the histone methyltransferase activity of MLL is crucial for transcriptional activation and downstream leukemogenesis.

In spite of their frequent association with poor prognostic AML, the lack of appropriate leukemia models has significantly hindered the progress in understanding the function of MLL-PTD and MLL amplification in leukemogenesis.\(^1,2\) While the relatively long latency and partial penetrance of AML in MLL\((40-50)^{\text{PTD}}\) mice indicate the requirement of additional events, such as genes co-amplified in the \(11q23\) amplicon\(^3\) for the development of full-blown leukemia, the study herein provides the first experimental evidence and novel insights into a cooperative transformation mechanism mediated by MLL-PTD and MLL amplification to induce leukemia in part by epigenetic deregulation of MLL downstream targets. Intriguingly, both gain-of-function and loss-of-function MLL mutations have been reported in acute leukemia, indicating that the appropriate activity of MLL is required for normal development, and its deregulation in either direction can lead to leukemogenic transformation.\(^1\) Instead of being a passive player in MLL-PTD leukemia, the study herein demonstrates the pathogenic functions and potential therapeutic value of targeting MLL amplification in acute leukemogenesis.

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