Acute myeloid leukemia induced by *MLL-ENL* is cured by oncogene ablation despite acquisition of complex genetic abnormalities

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**Running title:** Leukemic addiction to *MLL-ENL* expression

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Abstract

Chromosomal translocations involving 11q23 are frequent in infant acute leukemia and give rise to the formation of \textit{MLL}-fusion genes. The mechanism of leukemic transformation by these fusions has been the subject of numerous investigations. However, the dependence of acute leukemia on \textit{MLL}-fusion activity \textit{in vivo} and the efficacy of targeting this activity in order to eliminate disease have not been established. We have developed a model for conditional expression of \textit{MLL-ENL} in hematopoietic progenitor cells, in which expression of the fusion oncogene is turned off by doxycycline. Conditionally immortalized myeloblast cells derived from these progenitors were found to induce leukemia \textit{in vivo}. Leukemic cells isolated from primary recipient mice were shown to have acquired additional genetic abnormalities and when transplanted into secondary recipients induced leukemia with shortened latencies. However, the leukemic cells remained dependent upon \textit{MLL-ENL} expression \textit{in vitro} and \textit{in vivo} and its ablation resulted in regression of established leukemias. This study demonstrates that even genetically complex leukemias can be reversed upon inactivation of the initiating \textit{MLL}-fusion and has important implications for the design of novel leukemia therapies.
Introduction

A central goal of cancer research is the development of specific therapies for malignancies, which will avoid or minimize the toxicities associated with current treatment protocols. Development of such therapies relies on understanding the underlying biology of the cancer in question. An example of this approach is the study of MLL-fusion oncogenes in leukemia.\textsuperscript{1-4} These fusions are generated by chromosomal translocations of the MLL gene on chromosome 11q23,\textsuperscript{5} and are prevalent in infant acute lymphoid (ALL) and myeloid (AML) leukemia, and in treatment-related AML.\textsuperscript{6,7}

Several studies have addressed the importance of MLL-fusion activity for the initiation and maintenance of hematopoietic transformation. We and others have used conditional expression models to demonstrate that immortalization of cells transformed by MLL-ENL is abrogated \textit{in vitro} upon loss of fusion protein activity.\textsuperscript{8-10} Elegant studies have also shown that co-factors, such as Menin, and downstream transcriptional targets, such as Meis-1, Hoxa genes and Mef2c, are required for \textit{in vitro} transformation by certain MLL-fusions\textsuperscript{8,11-14} and in some cases leukemia engraftment.\textsuperscript{15} However, it remains to be demonstrated whether interfering with MLL-fusion activity will translate into elimination of established leukemia \textit{in vivo}. In this regard it is important to exclude that the bone marrow microenvironment may support the survival of leukemic stem cells in the absence of MLL-fusion activity and that acquisition of secondary mutations by leukemic cells \textit{in vivo} may render them resistant to MLL-fusion inhibition.

The importance of secondary mutations and genetic aberrations for the initiation and progression of acute leukemias associated with MLL translocations is not fully understood. The brief latencies of infant acute leukemias involving
MLL/11q23 rearrangements and their high concordance in monozygotic twins\textsuperscript{16} suggest that MLL-fusions may be sufficient to cause overt leukemia. This is consistent with the rapid onset of malignancy upon \textit{de novo} formation of the \textit{MLL-ENL} fusion in mice\textsuperscript{17} and leukemia induction by human cells expressing \textit{MLL}-fusion genes.\textsuperscript{18}

However, not all models of \textit{MLL}-fusion induced leukemia have short latencies and in some cases a pre-leukemic phase in disease progression has been documented.\textsuperscript{19,20} It has been suggested that the discrepancy between these models and leukemia in infants may be due to the MLL-fusions themselves rendering cells more susceptible to further DNA damage by genotoxic agents originally causing the \textit{MLL} translocation.\textsuperscript{21} This would result in the rapid acquisition of secondary mutations in cells expressing MLL-fusions. Furthermore, although a recent study detected very few additional genetic abnormalities in \textit{MLL}/11q23 rearranged ALL,\textsuperscript{22} analysis of \textit{MLL-ENL} positive ALL and AML found that half contained additional cytogenetic abnormalities, in most cases single trisomies.\textsuperscript{23}

This suggests that at least for some \textit{MLL}/11q23 rearranged leukemias, secondary mutations may be required for overt leukemia, and gives rise to the question of whether targeting MLL-fusion activity would be effective in these leukemias. The possibility that a small number of genes, for example the initiating oncogene, are required for maintaining malignancy despite the presence of numerous additional genetic abnormalities is suggested by the ‘oncogene addiction’ theory.\textsuperscript{24} Indeed, conditional \textit{Myc}\textsuperscript{25} and \textit{BCR-ABL}\textsuperscript{26} expression have been used to demonstrate regression of hematopoietic malignancies upon loss of oncogene expression. In this study, we have used conditional MLL-ENL expression to demonstrate that mice with established leukemias are cured by ablation of MLL-ENL expression \textit{in vivo}, despite
acquisition of secondary genetic abnormalities by the leukemic cells. These experiments suggest that targeting the transcriptional/signalling networks established by MLL-fusion oncogenes will lead to effective therapies for MLL/11q23 rearranged leukemias.
Methods

Mice

All mice were maintained in the animal facilities of the Institute of Child Health, London, and experiments were performed according to United Kingdom Home Office regulations and Institute of Child Health institutional guidelines.

Retroviral constructs and virus production

The pMSCV-MLL-ENL-neo and the pMSCV-tTA-IRES-EGFP were both constructed as described previously. The pMSCV-neo-TRE-MLL-ENL vector, containing the full-length flag-tagged MLL-ENL cDNA, was constructed by replacing the Myc-tagged TRE-mMLL fragment from the pMSCV-neo-TRE-mMLL-ENL vector with a flag-tagged TRE-MLL fragment. Retroviral supernatants were produced as described previously.

Isolation and infection of hematopoietic progenitor cells

Bone marrow hematopoietic progenitor cells (HPC) from C57BL/6 mice were purified by magnetic activated cell sorting (MACS) using the Lineage Cell Depletion Kit (Miltenyi Biotec, Surrey, United Kingdom). Lineage negative HPC were cultured for 24 hours in DMEM (Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated foetal calf serum (FCS, Sigma-Aldrich, Poole, United Kingdom), 100 U/ml Penicillin (Invitrogen), 100 μg/ml Streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen) (complete medium), 50 μM 2-mercaptoethanol (2-ME) (Sigma-Aldrich), 100 ng/ml stem cell factor (SCF), 10 ng/ml interleukin-3 (IL-3) and 10 ng/ml interleukin-6 (IL-6) (Peprotech, London, United Kingdom). HPC were then infected with retrovirus supplemented with the same growth factors on 2
consecutive days by spinoculation (centrifugation at 700 x g, 25°C, 45 minutes) in the presence of 5 μg/ml polybrene (Sigma-Aldrich), as described previously.\textsuperscript{10}

**Methylcellulose culture and generation of cell lines**

Colony forming assays were performed in Methocult M3434 (Stem Cell Technologies, Grenoble, France) supplemented with 10 ng/ml GM-CSF to generate immortalized cell lines, as described previously.\textsuperscript{10} Cell lines were maintained in RPMI-1640 (Invitrogen) with 10% FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-glutamine and 50 μM 2-ME supplemented with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. In some cases 2 μg/ml doxycycline (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) was added to the cultures *in vitro*. Colonies were stained with 1 mg/ml p-iodonitrotetrazolium (INT) (Sigma-Aldrich). The viability of cells after culture with different cytokine combinations (SCF, IL-3, IL-6 or GM-CSF) was measured using an MTS assay kit according to manufacturer’s instructions (Promega, Southampton, United Kingdom). Cytospins were made using a Shandon cytospin 3 (Thermo Electron Corporation, Essex, United Kingdom) and fixed and stained with Wright-Giemsa.

**Leukemogenesis assays**

Sublethally γ-irradiated (6.5 Gy) C57BL/6-CD45.1 mice were injected intravenously with 1 x 10\textsuperscript{6} immortalized cells. Mice were sacrificed when they developed clinical signs of disease. For secondary transplantation, sublethally irradiated C57BL/6-CD45.1 or C57BL/6 mice were injected intravenously with 1 x 10\textsuperscript{6} primary leukemic splenocytes. Peripheral blood was analysed for presence of leukemic cells at various time-points after transplantation. In some cases, following detection of leukemic cells
in the peripheral blood, recipient mice were administered 200 μg/ml doxycycline and 5% sucrose in the drinking water for a specified period of time. Mice were sacrificed when they developed clinical signs of disease, or at specified time-points after transplantation, and the liver, splenocytes and bone marrow cells harvested for analysis. Liver tissue was fixed in 10% buffered formalin (Sigma-Aldrich) and set in paraffin. Hematoxylin and eosin stain (H&E) was performed on 4μM sections.

**Flow cytometry**

Cells were stained using phycoerythrin (PE)- or allophycocyanin (APC)-conjugated antibodies to either c-Kit, Mac-1, Gr-1 or CD45.2 (eBioscience, Wembley, United Kingdom). Cells were resuspended in PBS, 0.5% bovine serum albumin and 0.05% sodium azide, and preincubated with unlabeled anti-Fcγ III/II receptor mAb (2.4G2), before staining with primary antibody. Flow cytometry was performed using a Cyan ADP analyser and Summit 4.3 software (Beckman Coulter, High Wycombe, United Kingdom).

**Southern blot analysis**

Genomic DNA was digested with BamHI. Southern blotting was performed according to standard protocols, using the 0.8-kb neomycin resistance gene cDNA labelled with \[^{32}\text{P}\] α-dCTP by random primer labelling.

**Cytogenetic analysis and comparative genomic hybridization**

10 μg/ml Colecemid (KaryoMAX, Invitrogen) was added to the cultures for at least 1 hour. Cells were then treated with 75 mM KCl, and cell pellets were fixed using a 3:1 methanol / acetic acid mixture. Chromosomes were visualised by Giemsa banding.
Array-based comparative genomic hybridization (aCGH), on genomic DNA isolated from immortalized and leukemic cells, was performed by Miltenyi Biotec using 4 x 44K Agilent Mouse Genome CGH Microarrays (Agilent Technologies, South Queensferry, United Kingdom). The original microarray data can be found in the ArrayExpress database under accession number E-MEXP-1911.
Real-time quantitative PCR

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen). RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). 2 μg RNA was converted into cDNA using M-MLV reverse transcriptase, amplification grade DNase I, random primers, RNAseOUT and dNTPs (all from Invitrogen), according to the manufacturer’s instructions. Real-time quantitative PCR was performed using TaqMan™ probe based chemistry and an ABI Prism 7900HT fast Sequence Detection System (Applied Biosystems, Warrington, United Kingdom). A specific primer and probe set spanning the \textit{MLL-ENL} breakpoint region was designed with PrimerExpress software v2.0 (Applied Biosystems) and used at 900 nM forward primer (5’-CAGGGTGTTTGCCTCTCTCTCTG-3’), 300 nM reverse primer (5’-GCGATGCCCCAGCTCTAA-3’), and 150 nM probe (5’-FAM-TGGACGTTGCACTCTACATGCCCAGC-TAMRA-3’). RNA expression levels of \textit{MLL-ENL}, \textit{Mpo} (Mm00447877_g1), \textit{Ltf} (Mm00434787_m1) and \textit{Mmp9} (Mm00600164_g1) were normalised to \textit{18S} RNA expression (Hs99999901_s1) and relative copy number of \textit{MLL-ENL} DNA was normalised to mouse \textit{β-Actin} DNA (4352933E). The $2^{ΔΔCT}$ relative quantitation method was used to determine the relative expression level or copy number of \textit{MLL-ENL}. All primer/probe sets were from Applied Biosystems.

Statistical analysis

Numbers of mice used in each experiment are provided in the figures or figure legends. Statistical analysis of survival curves was performed using the Mantel-Haenszel logrank test, where $P < 0.05$ was considered statistically significant.
Results

Conditional MLL-ENL immortalized cells induce leukemia

The retroviral expression constructs used in this study are depicted in Figure S1A. These constructs were used to generate immortalized myeloid cells with either conditional or constitutive expression of MLL-ENL (Figure S1B), as previously described. The ability of these cells to induce leukemia in vivo was assessed by transplanting them into sub-lethally irradiated congenic mice. The conditional and constitutive MLL-ENL immortalised cells induced AML in primary recipients with approximately the same latency (Figure 1A; Table 1). In order to assess if the disease could be transplanted, primary leukemic splenocytes were transplanted into sub-lethally irradiated secondary recipients. All secondary recipients developed AML with a much shorter latency than the primary recipients (Figure 1A; Table 1). They exhibited extensive leukemic cell infiltration in their livers (Figure S1C). Secondary leukemias derived from both conditional and constitutive immortalised cells expressed high levels of the myeloid marker Mac-1 and intermediate to high levels of the granulocyte marker Gr-1 (Figure 1B). This phenotype is very similar to that of the immortalised cells from which they were derived (data not shown).

An explanation for the shortened latency of the secondary recipients may be the outgrowth of a minor clone in vivo which was present in the original line but not detected by southern blot analysis. However, this does not appear to be the case since the leukemic cells had an identical retroviral integration pattern to the original immortalised cells (Figure 1C; Figure S1D). Alternatively, it is possible that the leukemic cells became ‘conditioned’ to the microenvironment in vivo such that upon secondary transplantation they were better able to respond to environmental signals and engrafted more efficiently. In order to test this hypothesis primary leukemic
splenocytes were either transplanted directly into secondary recipients or maintained in liquid culture for one month and then transplanted. Interestingly, the cultured leukemic cells did not lose the ability to induce AML with shortened latencies (Figure 2; Table 1). This result suggests that the decreased secondary latency is not simply due to an enhanced ability of the leukemic cells to respond to environmental signals in vivo.

Leukemic cells differentiate upon loss of MLL-ENL expression in vitro

Leukemic splenocytes were isolated from individual primary recipients and cultured in vitro. In all cases the leukemic cells proliferated more slowly than the immortalised cells from which they were derived (Figure S2A and data not shown). The cells remained growth factor dependent and shared the same growth factor requirements as the immortalised cells (Figure S2B). Both the conditional immortalised cells and their leukemic progeny ceased proliferating in vitro and failed to form colonies in methylcellulose upon inhibition of MLL-ENL expression by doxycycline treatment (Figure 3A,B). Furthermore, they exhibited changes in morphology and neutrophil granule gene expression consistent with terminal differentiation (Figure 3B,C). Leukemic cells differentiated at a similar rate and exhibited similar changes in cell surface marker expression as the immortalised cells (Figure 3D). As expected, the proliferation and phenotype of the constitutive immortalised cells and their leukemic progeny was not altered in response to doxycycline (data not shown).

Leukemic cells acquire genetic abnormalities in vivo

Cytogenetic analysis revealed that some leukemic cells had acquired gross chromosomal abnormalities which were not detected in the immortalised cells from
which they were derived. For example, the ME7 immortalised cells possessed a normal karyotype yet their leukemic progeny (ME7a) displayed clonal trisomy 6 (Figure 4A). In most cases the leukemic cells displayed more single cell abnormalities than the immortalised cells from which they were derived (Table S1).

More sensitive comparative genomic hybridisation (CGH) analysis was used to examine chromosomal copy number in the ME4b and ME7b leukemic cells, both of which appeared cytogenetically normal. This analysis revealed that ME4b leukemic cells had acquired an increase in copy number of a chromosome 4 region and a decrease in copy number of a chromosome 15 region, when compared to the parental ME4 immortalised cells (Figure 4B; Figure S3A). In addition, the ME7b leukemic cells possessed an increase in copy number of a small region of chromosome 6 (Figure S3B). These changes in copy number were not associated with changes in the copy number of the \textit{MLL-ENL} provirus (Figure S4A) or increased \textit{MLL-ENL} transcripts (Figure S4B).

**Continued MLL-ENL expression is required to maintain leukemia \textit{in vivo}**

It is possible that the acquired genetic abnormalities could confer resistance to loss of \textit{MLL-ENL} expression by the leukemic cells \textit{in vivo}. In order to examine this, secondary recipient mice with established leukemia were treated with doxycycline. The peripheral blood of secondary recipients was analysed at regular intervals after transplantation and doxycycline was administered following detection of leukemic cells. As expected, doxycycline had no effect on the accumulation of leukemic cells in the peripheral blood of mice transplanted with constitutive cells and did not alter their survival (Figure 5A). In contrast, doxycycline treatment of mice transplanted with conditional cells caused the complete elimination of leukemic cells from the
peripheral blood, in all cases examined (Figure 5A,B; Figure S5). Most of these mice remained healthy over the observation period studied (Figure 5A; Figure S5). Mice transplanted with conditional cells and left untreated, rapidly succumbed to fatal AML. The loss of conditional leukemic cells from peripheral blood following doxycycline treatment was most likely due to their terminal differentiation \textit{in vivo}. Thus, leukemic cells isolated from the spleen and bone marrow of doxycycline treated mice showed loss of c-Kit expression but no detectable apoptosis (Figure 6).

In some recipients, complete remission was not sustained and leukemic cells reappeared in the peripheral blood. All of the mice transplanted with ME4c leukemic cells relapsed while on doxycycline treatment (Figure S5). In addition, two of the ME4b recipients relapsed, one while on doxycycline treatment and one 41 days following doxycycline withdrawal (Figure S5). Since leukemias that relapsed on doxycycline demonstrated significant expression of \textit{MLL-ENL} mRNA, it is likely that doxycycline treatment selected for outgrowth of leukemic cells in which \textit{MLL-ENL} expression had become \textit{tTA}-independent (Figure S6A). This is also the most likely explanation for the ME4b relapse following doxycycline withdrawal (Figure S5). Leukemic cells from this mouse were able to form colonies in methylcellulose, but were slower growing and expressed lower levels of \textit{MLL-ENL} mRNA than cells from the ME4b recipient that relapsed while on doxycycline. Interestingly, \textit{MLL-ENL} expression in these leukemic cells was not abrogated by doxycycline treatment (Figure S6B).
**Discussion**

This study shows that immortalised myeloid cells with conditional expression of MLL-ENL gave rise to highly aggressive and invasive AML *in vivo* and that this disease regressed completely upon loss of MLL-ENL expression. Despite acquisition of additional genetic abnormalities, the leukemic cells remained dependent on continued MLL-ENL expression and in its absence underwent terminal myeloid differentiation *in vitro* and *in vivo*. Strikingly, treatment for as little as 10 days with doxycycline was sufficient to achieve complete remission in some cases, suggesting that the putative bone marrow leukemic stem cells were effectively targeted.

Previous studies have reported that leukemias initiated by MLL-fusions exhibit a reduced latency upon secondary transplantation.\(^{15,19,29}\) It is unclear whether this is due to acquired genetic abnormalities and epigenetic modification, or to enhanced engraftment potential resulting from *in vivo* conditioning to microenvironmental signals. We reasoned that the latter would be lost upon *in vitro* culture of leukemic cells. However, freshly isolated leukemic splenocytes were observed to give rise to AML in secondary recipients with similar latencies to those of cells transplanted following culture for one month *in vitro*. This data suggests that conditioning of leukemic cells does not account for the reduced secondary latencies in our model but are compatible with acquisition of heritable mutations during leukemia progression *in vivo*.

In several cases, we demonstrated that the leukemic cells had acquired additional genetic abnormalities, not present in the parental immortalised cells from which they were derived. These mutations included a trisomy of chromosome 6 in one case and gains and losses of chromosomal regions in a further two cases. This is consistent with cytogenetic analysis of human leukemias associated with the MLL-
ENL fusion. Whether these abnormalities are required for or merely a consequence of leukemic progression remains unclear. However, it is likely that the acquisition and selection of these abnormalities \textit{in vivo}, or of mutations which our analyses were not sensitive enough to detect, account for the reduced latency of AML in secondary recipients. The possible impact of secondary mutations on MLL-fusion induced leukemias, is illustrated by a study which found that co-expression of mutant FLT3, with an internal tandem duplication, with MLL-ENL in hematopoietic cells resulted in leukemias with considerably shortened latencies.

Induction of reversible hematopoietic malignancies has been demonstrated previously using the ‘Tet-Off’ system in which expression of the Myc and BCR-ABL oncogenes were controlled by a variety of different tTA transgenes. Experiments with Myc expressing lymphoid tumours demonstrated that although many tumors regressed upon prolonged Myc inactivation, others relapsed. Interestingly, all the relapsed tumours had additional chromosomal rearrangements and had become Myc-independent. Using a transgenic model in which tTA was placed under the control of the murine stem cell leukemia (SCL) gene regulatory elements, conditional expression of BCR-ABL resulted in reversible chronic myeloid leukemia (CML) and B lymphoid blast crisis. In these experiments, one of the leukemias was found to progress even during tetracycline treatment and on analysis of tumor cells was found to express BCR-ABL despite exposure to tetracycline. We found a similar loss of conditional oncogene expression in a few leukemias which relapsed, suggesting \textit{in vivo} selection for leukemic cells in which MLL-ENL expression had become doxycycline-insensitive.

In conclusion, in all of the leukemias induced by MLL-ENL, leukemic progression was entirely dependent upon continued \textit{MLL-ENL} expression. It is
important to note that the use of retroviral vectors in our study may have resulted in over-expression of the MLL-ENL oncogene. A recent study has demonstrated that the relatively low expression levels of the MLL-AF9 fusion achieved under the control of mouse MLL promoter elements, resulted in a reduced capacity of the fusion to transform committed progenitor cells. This suggests that co-operating secondary mutations may have a more important role in leukemic progression in AML patients, where the MLL-fusions are expressed under the control of endogenous promoters, than in our model. For these reasons, it will be important to examine the dependence of primary AML cells on expression of the appropriate MLL-fusion.

However, if extended to AML patient cells and to MLL-fusions in general, our data suggest that targeting MLL-fusion oncogenes, or downstream pathways regulated by them, will be effective at eradicating established leukemia. The paradigm for targeting the initiating oncogene in hematopoietic malignancy is treatment of BCR-ABL associated CML with the pharmacological ABL kinase inhibitor imatinib mesylate. This treatment is relatively non-toxic and has few side-effects. Interestingly, the addiction of these leukemias to BCR-ABL is underlined by the frequent presence of point mutations within BCR-ABL kinase domain, rendering it refractory to imatinib, in relapsed leukemia following imatinib therapy.

Unlike BCR-ABL, MLL-fusions regulate gene expression and it is unlikely that they can be targeted directly, because of the inherent difficulty in inhibiting transcription factor activity. A more promising approach would be to block downstream pathways regulated by MLL-fusions. The demonstration that inhibiting or ablating the expression of specific MLL-fusion target genes can reverse immortalization suggests that this may be feasible. We and others have recently demonstrated that native MLL is required for normal hematopoiesis and
hematopoietic stem cell activity.\textsuperscript{36,37} This suggests that specific therapies will have to be carefully calibrated against inhibition of normal MLL function, in order to avoid hematopoietic toxicity. However, the ‘oncogene addiction’ theory suggests that because of the novel or ‘bizarre’ circuitry set up by the initiating oncogene, leukemic cells may be more sensitive to inhibition of MLL activity than normal cells.\textsuperscript{38,39} This ‘addiction’ to high levels of oncogene activity probably explains why normal hematopoietic cells are not affected by ABL inhibition, whereas this results in elimination of CML cells.\textsuperscript{38}

In summary, this study demonstrates that leukemic cells are addicted to \textit{MLL-ENL} expression and suggests that targeting oncogenic pathways maintained by MLL-fusions in patients with 11q23 rearrangements would be a major therapeutic advance.
Acknowledgments

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Authorship

Contribution: S.J.H. and V.W-V. designed and performed experiments, analyzed and interpreted results and wrote the paper; S.J.C. performed cytogenetic analysis; N.J.S. performed the histopathology; J.deB. analyzed and interpreted results; O.W. supervised the project, designed and performed experiments, analyzed and interpreted results and wrote the paper.

S.J.H. and V.W-V. contributed equally to this paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.


References


Figure Legend

Figure 1. Leukemic cells with conditional expression of MLL-ENL induce disease with shortened latency in secondary recipients. (A) Survival curves of primary and secondary recipients transplanted with immortalized or leukemic myeloid cells. Immortalized myeloid cells with conditional (ME4, open circles) or constitutive (cME3, open diamonds) MLL-ENL expression were transplanted into primary recipient mice (n=4 for each group). The resulting leukemic cells were transplanted into secondary recipients (filled circles and diamonds, n=5 for each group). (B) Myeloid marker expression by leukemic cells. Dot plots show expression of CD45.2 and EGFP (left panel), and Gr1 and Mac1 (right panel), by leukemic ME4 and cME3 cells within the spleens of primary recipient mice. Numbers in the plots refer to the percentages of cells within each quadrant. (C) Immortalized cells and their leukemic derivatives show an identical pattern of retroviral integration. Southern blot analysis of genomic DNA isolated from immortalized cells ME4 and cME3 and their leukemic progeny ME4a, ME4b and cME3a. Blots show 5’ (ME4, 4a and 4b) and 3’ (cME3, 3a) end-fragments produced by digestion of the integrated provirus and genomic DNA with BamHI.

Figure 2. Leukemic cells retain the ability to induce accelerated leukemia after in vitro culture. Survival curves of mice transplanted with ME5a leukemic cells, either freshly isolated (open circles, n=10) or following 1 month culture in vitro (filled diamonds, n=10).
Figure 3. Differentiation of leukemic cells in response to doxycycline. (A) Fold accumulation ($\log_{10}$) in cell number of ME4 (squares), ME4a (triangles) and ME4b (diamonds) with (open symbols) or without (filled symbols) 2 $\mu$g/ml doxycycline. (B) Morphology of the cell lines (left panel, original magnification, x 400) and $p$-iodonitrotetrazolium stains of methylcellulose cultures (right panel) following culture with or without doxycycline for 4 and 8 days, respectively. (C) Relative level of neutrophil granule mRNA expression, measured by quantitative RT-PCR, in ME4 and ME4a cells after culture with (open bars) or without (black bars) doxycycline for 3 days. Plots show a decrease in primary granule myeloperoxidase (Mpo) and an increase in secondary granule lactotransferrin (Ltf) and tertiary granule matrix metallopeptidase 9 (Mmp9) gene expression following doxycycline treatment. Values for each gene were normalised to untreated ME4 cells. Columns represent the mean of triplicate measurements; error bars represent the standard deviations. (D) Changes in surface antigen expression in ME4 (left panel) and ME4a (right panel) cells following culture with or without doxycycline for 3 days. Upper panels show c-Kit and EGFP expression and lower panels, Gr-1 and Mac-1 expression. Numbers in the plots refer to the percentages of cells within each quadrant.

Figure 4. Leukemic cells have acquired genetic abnormalities. (A) Mouse GTG-banded karyotype of ME7a leukemic cells. Figure shows chromosomes arranged alongside their corresponding ideograms (original magnification x 1000). (B) Comparative genomic hybridization of ME4 and ME4b DNA samples. Figure illustrates the increase in copy number of a region of chromosome 4 (left panel) and decrease in copy number of a region of chromosome 15 (right panel) detected in ME4b leukemic cells when compared to ME4 immortalized cells. Each point on the
graph represents the log2 ratio value of a single oligonucleotide probe. The shaded area and vertical line indicate an aberrant region identified by the Z-score algorithm (CGH Analytics v3.4, Agilent Technologies).

**Figure 5. Abrogating MLL-ENL expression leads to elimination of established leukemias in vivo.** (A) Effect of doxycycline on survival of recipient mice (left panel) and elimination of leukemic cells from peripheral blood (right panel). Recipient mice were transplanted with the indicated leukemic cells and following their detection in the peripheral blood, one group was given doxycycline in their water (open diamonds; cME3a n=6, ME4a n=5, ME5a n=5, ME7c n=4) and the other left untreated (filled circles; cME3a n=4, ME4a n=5, ME5a n=5, ME7c n=5). Arrows indicate the point at which doxycycline treatment started and the bars on the survival curves indicate the length of treatment in each experiment. Doxycycline treated mice are still alive 350, 270 and 360 days after transplantation of ME4a, ME5a and ME7c cells, respectively. The graphs depict the presence of leukemic cells (Mac1⁺CD45.2⁺ cells in CD45.1 recipient mice for cME3a, and Mac1⁺EGFP⁺ cells for ME4a, ME5a and ME7a) as a percentage of total Mac1⁺ cells in the peripheral blood of recipient mice. Points on the graphs represent mean values and bars their standard deviations. (B) The presence of Mac1⁺EGFP⁺ leukemic ME4a cells in the peripheral blood of recipient mice before (day -1) and after (day 6 and 34) their treatment (right panel), or not (left panel), with doxycycline. Numbers in the top right quadrant represent Mac1⁺EGFP⁺ cells as a percentage of total Mac1⁺ cells. No mice in the untreated group survived until the last time-point shown, indicated by NS.
Figure 6. Leukemic cells differentiate in vivo in response to doxycycline. (A) Response of leukemic ME4b cells to doxycycline in vivo. Dot plots show Gr-1 and c-Kit expression (upper panel), and staining with 7AAD and Annexin V (lower panel), on gated EGFP+ ME4b cells in the bone marrow of mice 18 days after transplantation with ME4b cells and 3 days after their treatment (right plot), or not (left plot), with doxycycline. Numbers in the plots represent the percentage of cells in each quadrant (upper panel) and of apoptotic (7AAD–Annexin-V+) EGFP+ cells (lower panel). The mean fluorescence intensity of Gr-1 expression by ME4b cells (upper panel) increased from 684 to 1259 following doxycycline treatment. (B) Percentage of EGFP+ ME4b cells expressing c-Kit in the bone marrow and spleen of the recipient mice from A, treated (open bars) or not (filled bars) with doxycycline. Bars represent mean values (n=3) and error bars their standard deviations.
Table I. Latencies of primary and secondary leukemias

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<th>Leukemic cells</th>
<th>Latency of 1&lt;sup&gt;st&lt;/sup&gt; AML</th>
<th>Latency of 2&lt;sup&gt;nd&lt;/sup&gt; AML</th>
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<tr>
<td>ME4a</td>
<td>76</td>
<td>26.2 (±1.1, n=5)</td>
<td>17.8 (±9.2, n=5)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ME4b</td>
<td>83</td>
<td>26.8 (±0.5, n=5)</td>
<td>ND</td>
</tr>
<tr>
<td>ME4c</td>
<td>75</td>
<td>22.4 (±2.5, n=5)</td>
<td>ND</td>
</tr>
<tr>
<td>ME5a</td>
<td>83</td>
<td>16.6 (±2.2, n=10)</td>
<td>22.0 (±9.5, n=10)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ME7a</td>
<td>81</td>
<td>34.2 (±3.3, n=5)</td>
<td>14.2 (±3.3, n=5)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ME7b</td>
<td>89</td>
<td>30.5 (±2.4, n=4)</td>
<td>ND</td>
</tr>
<tr>
<td>ME7c</td>
<td>83</td>
<td>37.0 (±7.4, n=5)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Numbers represent the latency in days of the primary leukemias and the mean latency and standard deviation (numbers of mice per group are indicated) of secondary leukemias resulting from transplantation of the primary leukemias ex vivo or following culture for 1 month in vitro. ND = not determined.

<sup>a</sup>In vitro culture of leukemic cells caused a small but significant increase in ME5a latency (p=0.038, Mantel-Haenszel logrank test), had no effect on ME4a latency (p=0.269) and reduced the latency of ME7a (p=0.002).
Figure 1

A

% survival

1.0 74.1

2.5

ME4 2°

5.3 58.6

7.3

cME3 2°

B

C

4 4a 4b

3 3a

CD45.2

EGFP

Gr-1

Mac-1

Figure 1

0.1

3.4 48.0

9.6
Figure 2
Figure 3

A

B

C

D

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Figure 4
Figure 5
Figure 6

A

untreated

dox

Gr-1

c-Kit

7AAD

Annexin-V

B

% c-Kit^+ cells

BM

SP

untreated
dox

n=3

n=3
Acute myeloid leukemia induced by MLL-ENL is cured by oncogene ablation despite acquisition of complex genetic abnormalities

Sarah J Horton, Vanessa Walf-Vorderwulbecke, Steve J Chatters, Neil J Sebire, Jasper de Boer and Owen Williams