Preclinical modeling of cytosine arabinoside response in *Mll-Enl* translocator mouse leukemias

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Abstract

Mouse models of human cancer are a potential preclinical setting for drug testing and for development of methods for delivery of macromolecular drugs to tumors. We have assessed a mouse model of leukemia caused by Mll-Enl protein fusion as a preclinical situation in which myeloid-lineage leukemia results from *de novo* occurrence of chromosomal translocations between *Mll* and *Enl* genes. Here, we show that the mouse leukemias respond to cytosine arabinoside, a frontline treatment for human leukemia. The observations show that the myeloid cells are susceptible to the drug and the mice undergo a remission that comprises a reduction of the myeloid population of cells and recovery of the lymphoid population. This translocator model should therefore prove useful for future drug assessments against the recurrent mixed-lineage leukemia–associated translocations. [Mol Cancer Ther 2008;7(3):730–5]

Introduction

Mouse models of human cancers are important for developing rational therapeutic strategies and for preclinical drug testing. In addition, new molecular-based therapeutics require special preclinical testing as delivery of these macromolecules to tumors presents a continuing major challenge. Mouse preclinical models have been criticized because some compounds that seemed to be therapeutic when tested in xenograft models failed to be efficient in humans (1). In fact, the number of anticancer drugs that fail in the clinic far outweighs those considered effective in preclinical trials (2). This suggests that the models used for testing potential anticancer therapeutics are imperfect and that the selection procedure for progression of molecules into the clinic requires improvement. The development of more faithful genetically engineered mouse models promises to address some of these discrepancies. These should allow a more biologically appropriate model for testing novel anticancer therapeutic agents. These new models could potentially be useful to test drug efficacy as well as variables of delivery, toxicity, specificity, and effectiveness of the new drug before use in clinical settings.

Many human tumors have reciprocal chromosomal translocations as an underlying cause. Among the most frequent target of translocation in human leukemias is the mixed-lineage leukemia (*MLL*) gene (3). *MLL* gene abnormalities are found in both childhood (~10% of all pediatric leukemias) and adult leukemias (~5% of acute leukemias; ref. 4). Around 5% to 10% of all *MLL*-associated leukemias are therapy related as a side effect of treatment with topoisomerase II targeting drugs or of other treatments including radiotherapy (5). The many MLL fusion partners that have been identified to date (>50; ref. 6) represent a structurally heterogeneous group of proteins. The most frequent partners are AF4, AF9, and ENL resulting from t(4;11), t(9;11), or t(11;19).

We have generated previously a translocator mouse model in which *de novo Mll-Enl* reciprocal chromosomal translocations occur conditionally by means of Cre-*loxP*-mediated recombination (7) and these translocator mice develop leukemias. In one line of mice, the Cre recombinase expression was governed by the *Lmo2* promoter, allowing Cre expression in pluripotent hematopoietic stem cells. This model resulted in a myeloproliferative-like acute myeloid leukemia (8) with a rapid onset and high penetrance of leukemogenesis. This translocator model provides a possible preclinical model for novel drug testing or for assessing untried combinations for current drugs. To assess the predictive value and validate the *Mll; Enl; Lmo2-Cre* translocator mouse model as a preclinical model, leukemic mice were treated with cytarabine (cytosine arabinoside or Ara-C), one of the most important compounds used in chemotherapy regimes of patients with acute myeloid leukemia (9), including *MLL* translocation-associated leukemias. *Mll; Enl; Lmo2-Cre* translocator mice treated with Ara-C showed a reduction of myeloblasts (leukemic cells) in blood and spleen, with restoration of lymphocyte counts to normal levels. Furthermore, a reduction in the size of the neoplastic organs to normal levels was observed. Thus, as in most patients with acute myeloid leukemia, these translocator mice achieve a remission of the disease in response to treatment with

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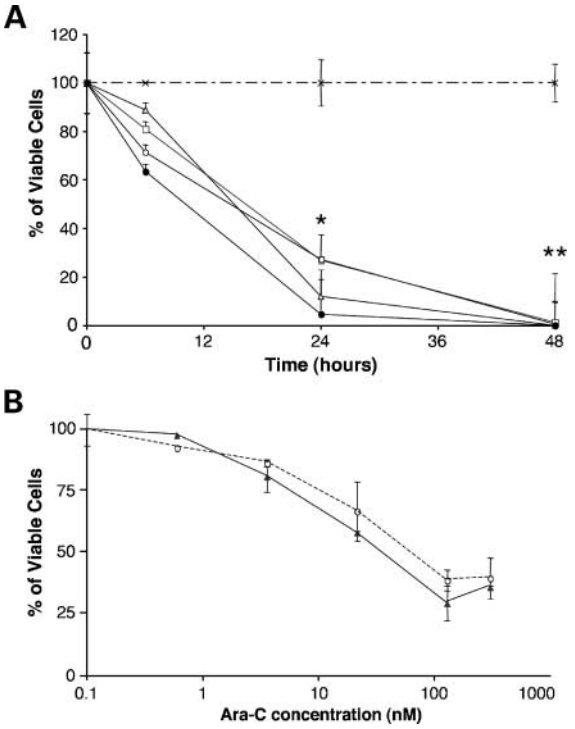

Figure 1. Cytotoxicity assays with Ara-C of *Mll; Enl; Lmo2-Cre* cell cultures. *Mll; Enl; Lmo2-Cre* mice with leukemia were donors for cell lines made from myeloid cells from the infiltrated spleens. Dose-response and time-response effect of Ara-C in *Mll; Enl; Lmo2-Cre* cell cultures. **A,** 5×10^5 cells were plated in 24-well plates (in triplicate) and incubated in the presence of 0 (x), 0.1 (□), 0.3 (■), 0.6 (△), and 1 (●) μmol/L Ara-C for 6, 24, and 48 h. After incubation, cells were harvested and viable cell numbers were calculated by trypan blue exclusion. Cell counts were obtained in duplicate. Mean ± SD. *, $P < 0.05$; **, $P < 0.01$. **B,** dose-response curve of *Mll; Enl; Lmo2-Cre* to Ara-C at 24 h. 5×10^5 cells were plated in 24-well plates (in triplicate) and incubated in the presence of 0 to 300 nmol/L Ara-C. After 24 h, cells were harvested and viable cell numbers were calculated by trypan blue exclusion. Exclusion assays were conducted in duplicate. Mean ± SD. The IC_{50} (50% inhibitory concentration) for the *Mll; Enl; Lmo2-Cre* cell line (▲) was 56 nmol/L. Results are compared with a primary spleen cell culture (o) as a control.

Ara-C. These results highlight the faithfulness of this model to the human MLL-ENL myeloid leukemia and support its use as a new model for preclinical studies.

Materials and Methods

Translocator Mouse Strains

Mouse lines carrying *loxP* sites inserted in *Mll* and *Enl* introns have been described (7, 10). Mice expressing Cre recombinase under the control of *Lmo2* (11) were bred with those homozygous for both *Mll* and *Enl loxP* alleles (mice carrying *Mll* and *Enl* with *loxP* sites with or without a *Lmo2-Cre* allele are designated as *Mll; Enl; Lmo2-Cre* or *Mll; Enl*, respectively).

Cytotoxicity Assay in Cell Cultures

Cell lines from tumors of *Mll; Enl; Lmo2-Cre* mice were described previously (7). For cytotoxicity assays, 5×10^5 cells were plated (in triplicates) in 24-well plates and incubated in growth medium containing 0 to 1 μmol/L cytarabine (Ara-C; David Bull Laboratories) for 6, 24, and 48 h. After incubation, viable cell numbers were calculated by adding 20 μL cell culture to 20 μL trypan blue (0.4%, w/v), after which viable cells were counted based on the presence (dead cells) or exclusion (live cells) of the dye. Cell counts were conducted in duplicate. Results are shown as mean ± SD.

In vivo Treatment of Translocators with Ara-C

From ages 3 to 4 weeks, *Mll; Enl; Lmo2-Cre* or control mice had blood samples taken every 2 days as required. Blood samples were obtained before administration of Ara-C. Leukemia was monitored by increase in circulating leukemic (granuclocyte) cells in blood and considered positive when granulocyte counts exceeded 6×10^3 cells/mm^3. Leukemic mice were injected i.p. with doses of Ara-C ranging from 0 to 100 mg/kg (resuspended in 100 μL sterile PBS) for 5 consecutive days unless otherwise stated. Blood samples were collected every 2 days and analyzed in a Scil vet abc counter (Scil Animal Care Technologies). Blood smears were stained with May-Grünwald-Giemsa stain. The low-dose experiments (30 mg/kg) consisted of two rounds of 4 consecutive days of injection followed by a 3-day injection-free interval.

At the termination point of the experiments, mice were culled and relevant tissues were removed. Whole animals and the removed tissues were weighed. Flow cytometric analysis was conducted to determine the surface protein expression phenotype of the tumors using a FACSCalibur

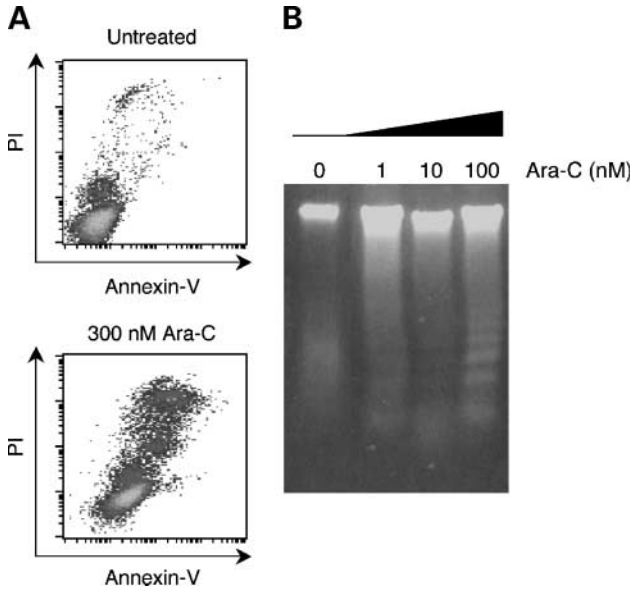

Figure 2. Ara-C induces apoptosis in *Mll; Enl; Lmo2-Cre* cell culture. **A,** 5×10^5 myeloid cells (from the infiltrated spleens of *Mll; Enl; Lmo2-Cre* leukemic mice) were incubated in 24-well plates (in triplicate) with or without 300 nmol/L Ara-C for 24 h before assay for apoptosis by flow cytometry analysis of Annexin V-FITC binding. Data show FITC signal and propidium iodide (*PI*) uptake levels. **B,** DNA fragmentation assay. After 24-h incubation with Ara-C ranging from 0 to 100 nmol/L, 5×10^6 cells were harvested and DNA was prepared. Samples were subjected to electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

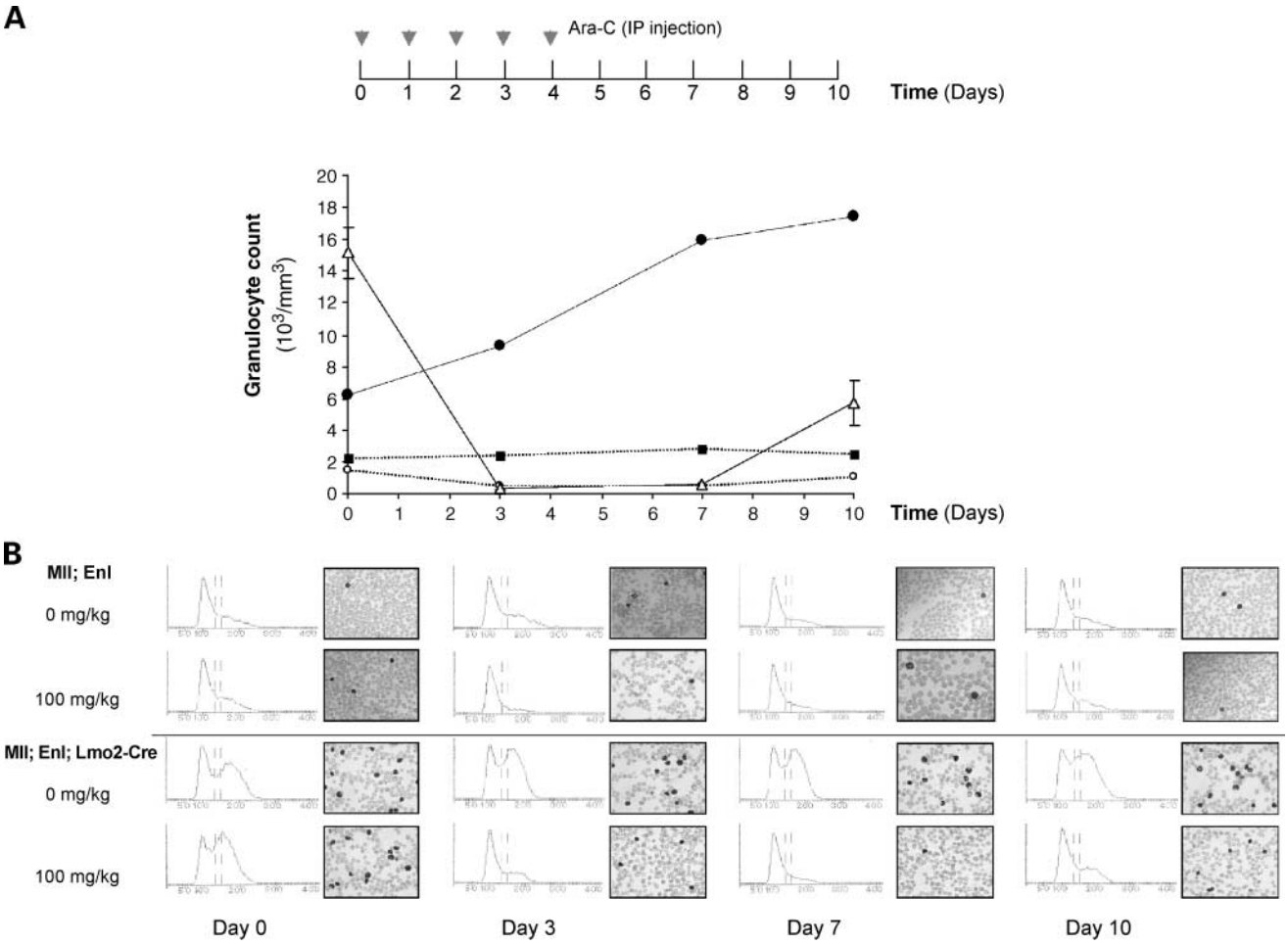

Figure 3. *In vivo* effect of Ara-C in *Mll; Enl; Lmo2-Cre* leukemic mice. *Mll; Enl; Lmo2-Cre* (leukemic) or *Mll; Enl* mice were injected i.p. with 0 (PBS vehicle only) or 100 mg/kg Ara-C for 5 consecutive days. **A,** granulocyte blood levels were monitored for 10 d starting before injection (day 0) and after every 3 d. Quantification of granulocyte levels in the blood is shown for untreated and Ara-C-treated *Mll; Enl* mice and *Mll; Enl; Lmo2-Cre* leukemic mice. *Mll; Enl* mice: untreated (PBS vehicle only) (■); treated with 100 mg/kg Ara-C (○). *Mll; Enl; Lmo2-Cre* mice: untreated (vehicle only) (●); treated with 100 mg/kg Ara-C (△). **B,** leukemia monitoring by blood sample analysis at day 0 and at 3, 7, or 10 d after injection of vehicle or 100 mg/kg Ara-C. Coulter counter profiles [forward scatter (*FSC*) versus cell counts] and the corresponding blood smears (×40) stained with May-Grünwald-Giemsa for each indicated *Mll; Enl* or *Mll; Enl; Lmo2-Cre* mouse group are shown.

with fluorescent antibodies. For this analysis, cell suspensions (100 μL) from spleen were incubated with the appropriate antibodies for 1 h on ice, washed with PBS, and analyzed using FACSCalibur. The antibodies used (PharMingen; BD Biosciences) were PE-labeled anti-Mac-1(CD11b), FITC-labeled anti-Gr-1(Ly-6G), PE-labeled anti-CD4(L3T4), FITC-labeled anti-CD8a, PE-labeled anti-Thy1.2, and FITC-labeled anti-B220(CD45R). The appropriate isotype controls were used for each antibody (Ig2aκ for anti-B220, anti-Thy1.2, and anti-CD4 and Ig2bκ for anti-Mac-1, anti-Gr-1, and anti-CD8a).

Results

Sensitivity of *Mll; Enl; Lmo2-Cre* Cells to Ara-C *In vitro*

To evaluate if the *Mll; Enl; Lmo2-Cre* translocator mice would be responsive to Ara-C treatment, we analyzed *in vitro* cytotoxicity assays. *Mll; Enl; Lmo2-Cre* cell cultures were established from splenic infiltrates of myeloid leukemia cells (7). These cells were incubated in growth medium containing varying amounts of Ara-C for 6, 24, or 48 h. After treatment, viable cell numbers were calculated using trypan blue exclusion. Figure 1A shows the time-response curve of *Mll; Enl; Lmo2-Cre* cells when exposed to increasing concentrations of Ara-C. A reduction of cell viability to 65% was observed as early as 6 h of culture in the presence of 1 μmol/L Ara-C. Even when incubated with 0.1 μmol/L Ara-C, there was an almost complete depletion of viable cells after 48 h of treatment. Figure 1B shows the dose-response curve of *Mll; Enl; Lmo2-Cre* cells to Ara-C treatment at 24 h. The IC_{50} (half-maximal inhibitory concentration) for the *Mll; Enl; Lmo2-Cre* cell line was calculated as 56 nmol/L. Similar results were obtained with primary spleen

cultures and parallel to those seen for Ara-C treatment of established cell lines (12).

The mechanism of cell death induced by Ara-C is apoptosis (13). This was confirmed as the killing mechanism for the *Mll-Enl* translocator cells. Cultures of cells were grown in the presence or absence of 300 nmol/L Ara-C for 24 h, and flow cytometry detection of externalized phosphatidylserine was done using Annexin V-FITC binding as a measure of cells dying by apoptosis (Fig. 2A). A population of 30% of apoptotic cells binds the Annexin V

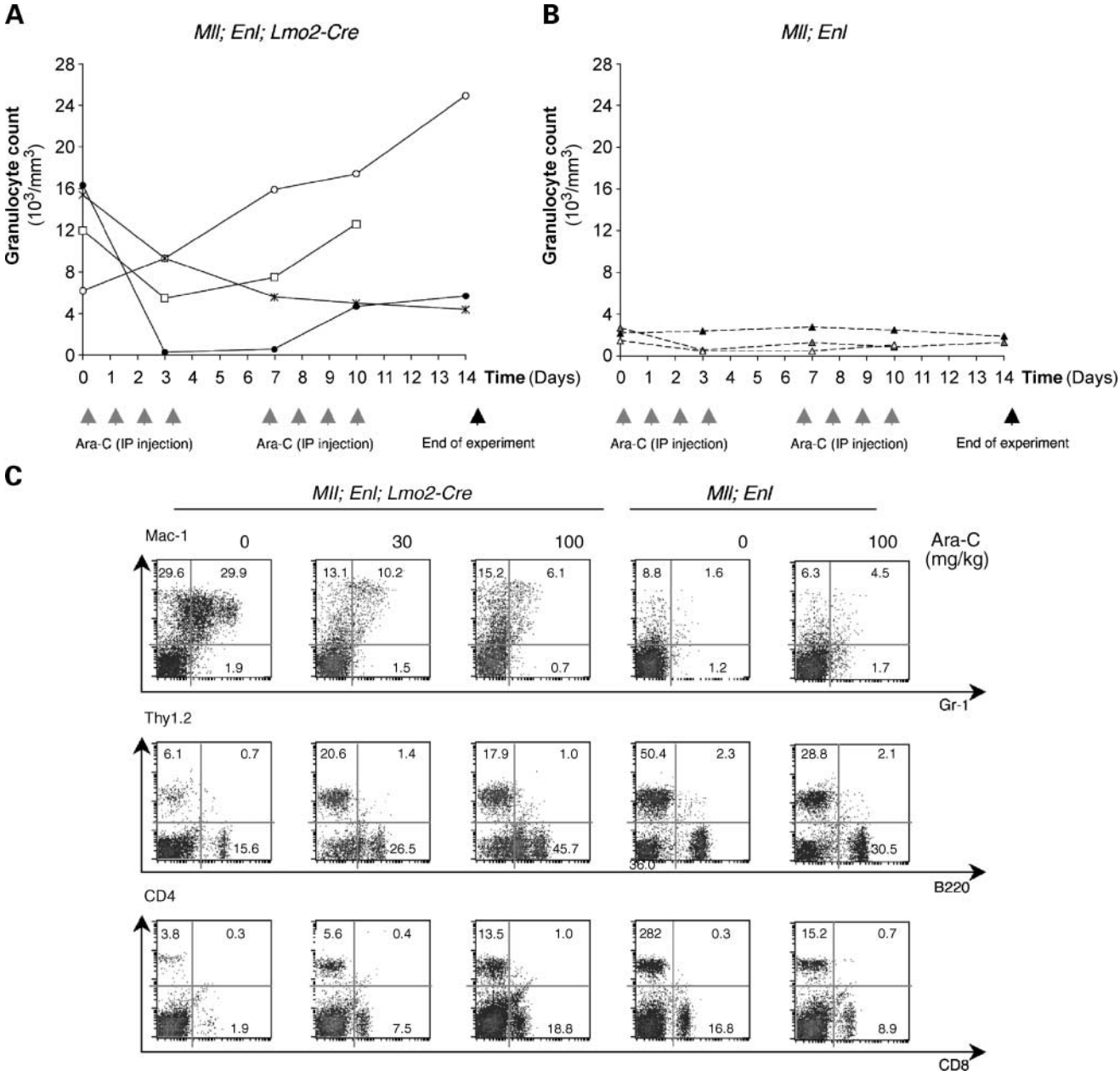

Figure 4. Phenotypic disruption by Ara-C treatment of *Mll; Enl; Lmo2-Cre* leukemic mice. *Mll; Enl; Lmo2-Cre* (leukemic) or *Mll; Enl* mice were injected with 0 (vehicle only), 3.5, 30, or 100 mg/kg Ara-C in two rounds of 4 consecutive days of injection separated by a 3-day injection-free interval. Granulocyte blood levels were determined before treatment (day 0) and at days 3, 7, 10, and 14 after treatment by Coulter counting. Leukemia was considered to be present when granulocyte counts exceeded 6×10^3 cells/mm^3. **A,** quantification of granulocyte levels in the blood are shown for untreated or Ara-C-treated *Mll; Enl; Lmo2-Cre* mice. Untreated (○); treated with 3.5 mg/kg (□), 30 mg/kg (*) or 100 mg/kg (●) Ara-C. **B,** quantification of granulocyte levels in the blood for untreated or Ara-C-treated *Mll; Enl* mice. Untreated (▲); treated with 30 mg/kg (▲) or 100 mg/kg (△) Ara-C. **C,** decrease in myeloid (Mac-1/Gr-1 positive) tumor cells in response to treatment with Ara-C. Flow cytometric analysis of surface antigen expression (either Mac-1 + Gr-1, Thy1.2 + B220, or CD4 + CD8) of spleen cells of mice after two rounds of treatment with Ara-C (30 or 100 mg/kg for *Mll; Enl; Lmo2-Cre* mice or only 100 mg/kg for *Mll; Enl* only mice).

after Ara-C treatment. This was confirmed by analyzing DNA laddering (caused by intrinsic nuclease cleavage of chromatin beads) following 24-h treatment with 100 nmol/L Ara-C (Fig. 2B).

Ara-C Can Achieve Tumor Remission in *Mll; Enl; Lmo2-Cre* Leukemic Mice

The effects of Ara-C on leukemia *in vivo* was tested using the *Mll-Enl* translocator mice. Litters were selected from crosses between homozygous *Mll; Enl* and heterozygous *Lmo2-Cre* mice giving a mixture of pups with either *Mll; Enl*; *Lmo2-Cre or Mll; Enl* genotypes. Before drug treatment, blood leukocyte levels were determined in the *Mll; Enl; Lmo2-Cre* and *Mll; Enl* mice starting 2 weeks after birth and following leukemia by blood granuclocyte levels.

Sensitivity of leukemic *Mll; Enl; Lmo2-Cre* mice to Ara-C was tested with a high-dose regimen. Leukemic mice (age 3-4 weeks) were injected i.p. with PBS as vehicle (three mice) or 100 mg/kg Ara-C for 5 consecutive days (five mice). Blood samples were collected every 3 days and analyzed in the Coulter counter. Figure 3A shows an example for each group. A decrease in granulocyte levels was observed in the blood immediately after treatment with 100 mg/kg Ara-C (Fig. 3A). By day 3 of the experiment, granulocyte blood levels achieved normal (nonleukemic) values of $\sim 1.2 \times 10^3$ to 1.5×10^3 cells/mm^3. After treatment, granulocyte blood levels remained within normal levels for the experimental period (reflected by the death of the control leukemic mouse that had received vehicle only). The dynamics of the blood leukemic cells is shown in Fig. 3B, comparing leukocyte counts and smears of an *Mll; Enl* and *Mll; Enl; Lmo2-Cre* mice with and without 100 mg/kg Ara-C treatment. The leukemic cells dominate in untreated mice, resulting in death within ~ 2 weeks, whereas Ara-C treatment causes leukemia remission over the allowed period of treatment.

An alternative experimental protocol was investigated in which two consecutive rounds of Ara-C treatment were given, using 4 days of injection followed by a 3-day injection-free interval, and subsequently by a further 4 days of dosing. In this approach, different Ara-C concentrations ranging from 3.5 to 100 mg/kg were assessed. As seen in Fig. 4A and B, lower doses (3.5 mg/kg) were not sufficient to deplete completely granulocyte levels in blood, and when treatment was withdrawn, most of the mice relapsed. However, higher doses (30 and 100 mg/kg) successfully decreased leukemic cells in blood to normal levels, sustained through the time of leukemia progression of untreated or 3.5 mg/kg treated mice.

Ara-C Specificity Causes Neoplastic Tumor Regression

The specific consequences for the leukocyte subpopulations were assessed using flow cytometry (Fig. 4C). As the *Mll; Enl; Lmo2-Cre* translocator mice show splenomegaly as a feature of the leukemia, at the termination point of the experiments, mice were culled and spleens were removed and weighed. Ara-C treatment achieved a reduction of spleen size (neoplastic tissue) to normal values (Supplementary Fig. S1).[4] Further, spleen cells were prepared as single-cell suspension for flow cytometric analysis (Fig. 4C). Myeloid (Mac-1/Gr-1)–positive cells dramatically decreased in response to Ara-C treatment, whereas B cells (B220 positive) or T cells (Thy1, CD4, or CD8 positive) appear approximately normal after the Ara-C regimen. However, completely normal levels of granulocytes could not be sustained in this experimental protocol, suggesting that a maintenance therapy would be needed to achieve long-term remission and to avoid relapse. This is not surprising because Ara-C treatment, as well as most of the currently used therapeutics, may be effective at killing most leukemic cells but not capable of eliminating all of them. In these regards, this mouse model behaves similarly to human patients, most of whom, without any additional post-remission therapy, have a high probability of relapse (9).

Discussion

Ideally, mouse models of human cancer should have short tumor latency and high penetrance, be an authentic replication of the corresponding human tumor, and have spontaneous occurrence due to the presence of a single alteration in the mouse genome. Models in which most or all of a cohort develop tumors in a short time provide a cost-effective preclinical model. The *Mll; Enl; Lmo2-Cre* translocator model fulfills most of these criteria because all mice of the *Mll; Enl; Lmo2-Cre* genotype die within 120 days and leukemia (blood granulocytes) can be observed as early as 3 to 4 weeks (7).

We have shown that the *Mll; Enl; Lmo2-Cre* translocator model responds to Ara-C chemotherapy similarly to humans. As in most patients with acute myeloid leukemia, mice achieve remission characterized by cytoreduction of blast cells in blood and spleen, as well as a reduction in size of the neoplastic organ (in this case, the spleen). However, without additional postremission therapy, most of the mice relapsed as is also the case with most human patients. These results highlight the faithfulness of this model to the human *MLL-ENL* myeloid leukemia and support its usage as a new tool in preclinical studies. Practical improvements to the model could be introduced to increase the cost-effectiveness of the model even further. One of these options would be to include a luciferase reporter in cells that have a translocation to allow bioluminescent imaging *in vivo* to follow tumor response to therapy. A further feature of value would be a conditional form of Cre expression. We have developed an *Lmo2-CreERT2* strain of mice[5] and these will allow a synchronized activation of translocations in a *Mll; Enl; Lmo2-CreERT2* strain. Breeding these mice with Cre-dependent expression of luciferase will give a fully conditional, image-able model for *MLL*-associated leukemias.

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
[5] R. Pannel and T.H. Rabitts, unpublished data.

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References

1. Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. Nat Rev Drug Discov 2006;5:741 – 54.

2. Suggitt M, Bibby MC. 50 years of preclinical anticancer drug screening: empirical to target-driven approaches. Clin Cancer Res 2005;11:971 – 81.

3. Ayton PM, Cleary ML. Molecular mechanisms of leukemogenesis mediated by MLL fusion proteins. Oncogene Rev 2001;20:5695 – 707.

4. Look AT. Oncogenic transcription factors in the human acute leukemias. Science 1997;278:1059 – 65.

5. Pui C-H, Behm FG, Raimondi SC, et al. Secondary acute myeloid leukemia in children treated for acute lymphoid leukemia. N Eng J Med 1989;321:136 – 42.

6. Huret JL, Dessen P, Bernheim A. An atlas of chromosomes in hematological malignancies. Example: 11q23 and MLL. Leukaemia 2001;15:987 – 99.

7. Forster A, Pannell R, Drynan LF, et al. Engineering *de novo* reciprocal chromosomal translocations associated with Mll to replicate primary events of human cancer. Cancer Cell 2003;3:449 – 58.

8. Kogan SC, Ward JM, Anver MR, et al. Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. Blood 2002;100:238 – 45.

9. Burnett AK. The treatment of AML: current status and novel approaches. Haematology 2005;10:50 – 3.

10. Collins EC, Pannell R, Simpson EM, Forster A, Rabbitts TH. Inter-chromosomal recombination of Mll and Af9 genes mediated by Cre-loxP in mouse development. EMBO Rep 2000;1:127 – 32.

11. McCormack MP, Forster A, Drynan LF, Pannell R, Rabbitts TH. The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. Mol Cell Biol 2003;3:9003 – 13.

12. Crisp LB, Smith SM, Mathers MA, Young GA, Lyons SD, Christopherson RI. Effects of cytosine arabinoside on human leukemia cells. Int J Biochem Cell Biol 1996;28:1061 – 9.

13. de Vries JF, Falkenburg JH, Willemze R, Barge RM. The mechanisms of Ara-C-induced apoptosis of resting B-chronic lymphocytic leukemia cells. Haematologica 2006;91:912 – 9.