A human genome editing-based MLL::AF4 B-cell ALL model recapitulates key cellular and molecular leukemogenic features

Tracking no: BLD-2023-020858R1

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Abstract:
The cellular ontogeny and location of the MLL-breakpoint influence the capacity of MLL-edited CD34+ HSPCs to initiate pro-B-ALL, and recapitulate the molecular features of MLL-AF4+ infant B-ALL patients. We provide key insights into the cellular-molecular leukemogenic determinants of MLL-AF4+ infant B-ALL.

Conflict of interest: No COI declared

COI notes: PM is founder of the spin-off OneChain Immunotherapeutics which has no connection with the present research. The remaining authors declare no competing interests.

Preprint server: No;

Author contributions and disclosures: CB, RT-R, TV-H, OM, PP, AM, VR, MV, SC, NG-G, SR-P, JCS, QO-B ALS and JRT performed experiments and interpreted data. OW, MFF, AR, CM, RM, TAM, and PM supervised research and contributed key knowledge, techics and reagents. CB, RT-R and PM conceived the study and funded the research. All authors have read and agreed to publish the manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Reagents/protocols will be provided to other investigators upon contact with the corresponding author.

Clinical trial registration information (if any):
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Running title: A novel CRISPR-Cas9-based disease model reveals leukemogenic determinants of MLL::AF4 B-ALL.

Keywords: MLL::AF4, infant B-ALL, MLL breakpoints, cord blood, fetal liver, CD34+ HSPCs.

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Abstract

The cellular ontogeny and location of the MLL-breakpoint influence the capacity of MLL-edited CD34+ HSPCs to initiate pro-B-ALL, and recapitulate the molecular features of MLL-AF4+ infant B-ALL patients. We provide key insights into the cellular-molecular leukemogenic determinants of MLL-AF4+ infant B-ALL.

Chromosomal translocations involving the mixed lineage leukemia (MLL) gene drive leukemia development; key determinants of disease outcome are patient age, MLL fusion partner, and cell-of-origin. The MLL::AF4 (also known as KMT2A::AFF1) fusion protein (MA4) is present in 80% of infants with B-cell acute lymphoblastic leukemia (iB-ALL) and is associated with prenatal origin and poor prognosis. In fact, the MA4 fusion occurs in early hematopoietic stem/progenitor cells (HSPCs) and is a causal oncogenic driver in iB-ALL. The prevalence of the MA4 fusion is higher in infant than in non-infant B-ALL patients and is associated to higher frequency of refractoriness, relapse and poor prognosis transcriptomic signatures. Molecularly, the distribution of genomic breakpoints within the MLL breakpoint cluster region (BCR) varies significantly, with a notable telomeric hotspot in infants and a preference for centromeric breakpoints in non-infants. Overall, these findings support the notion that age-related mechanisms and inherent biological differences contribute to MA4+ B-ALL. Studying these mechanisms is, however, challenging due to the dearth of human cellular models. CRISPR-Cas9 gene editing was recently used to recreate the t(4;11)/MLL::AF4 translocation in human HSPCs, mimicking the characteristics of the disease, but the impact of the cell-of-origin and its functional consequences were not compared, nor was the potential to induce pro-B-ALL in vivo determined. Here, we compared the molecular impact and leukemogenic potential of both centromeric (intron 10, MLL\textsuperscript{i10}) and telomeric (intron 12, MLL\textsuperscript{i12}) MLL breakpoints in human CD34+ HSPCs from different developmental stages: second trimester prenatal fetal liver (FL) and postnatal cord blood (CB).

We designed and tested several single-guide RNAs (sgRNAs) to target intron 10 (centromeric) or intron 12 (telomeric) of MLL, along with AF4 intron 3 (Figure 1A, Table S1). Nucleofected FL and CB cells were cultured with cytokines to promote MLL-edited cell expansion (Figure 1B). In contrast to mock cells, edited cells showed exponential growth starting at day 20–25 post-nucleofection, resulting in billions of MA4-immortalized cells irrespective of their origin (Figure 1C, S1A-B). Most cells in both cultures harbored MLL rearrangements (Figure 1D, S1C), although the emergence of MLL\textsuperscript{i12}-rearranged cultures was consistently slower than MLL\textsuperscript{i10}-rearranged cultures (Figure 1E, S1D). We confirmed the presence of the chromosomal translocation, the fusion gene RNA transcript (Figure S1D,E), and the chimeric protein (Figure S1F). Furthermore, we found that targeting either intronic region equally immortalized in vitro both prenatal and neonatal CD34+ cells.

We confirmed similar MA4 expression levels between CRISPR-edited cells and patient-derived cells (Figure S2A). Transcriptome analysis revealed distinct clustering patterns of MLL-edited...
HSPCs according to the location of the MLL breakpoint (centromeric-Mi10A4 versus telomeric-Mi12A4) irrespective of the cellular ontogeny (FL or CB) (Figure S2B). Specifically, the expression of HOX cluster genes, known to be involved in MA4-mediated pathogenesis15,16, exhibited variation dependent on the MLL breakpoint location rather than the tissue of origin (Figure 2A). Notably, crucial HOX/MEIS genes such as HOXA9, HOXA10, and MEIS1 showed significant upregulation in FL and CB Mi10A4-edited cells but not in Mi12A4-edited cells (Figure S2C). We performed ChIP-seq analysis to understand how HOXA9, HOXA10 and MEIS1 expression is regulated in gene edited-derived cells, which revealed specific MA4 binding at the promoter and spreading into the gene bodies of HOXA9, HOXA10 and MEIS1 in Mi10A4-edited FL and CB CD34+ cells but not in Mi12A4-edited cells (Figure 2B). Notably, no significant differences were observed in other epigenetic marks across all edited cells. Collectively, these findings confirm the transcriptional regulatory capacity of Mi10A4, but not Mi12A4, in regulating the expression of HOXA9, HOXA10 and MEIS1 through direct and specific binding to their regulatory regions. This recapitulates the HOXAlow transcriptomic signature reported in infant patients3,15,17.

The MA4+-edited cells exhibited in vitro expansion with a CD45+CD33+CD34-CD19- myeloid phenotype (Figure 1). Therefore, we next examined the myeloid clonogenic potential of MLL-edited FL and CB CD34+-HSPCs. Both mock-targeted and edited cells showed similar primary colony-forming unit (CFU) potential and sustained expression of MA4 (Figures S2D-E); however, only MLLi10-edited cells displayed clonogenic capacity upon serial replating, regardless of their cellular ontogeny (Figures S2D-E). Notably, these results align with clinical data18 indicating a higher prevalence of acute myeloid leukemia (AML) in patients with a centromeric (42%) rather than a telomeric (30%) MLL breakpoint (Figure S2F).

We next investigated the impact of cellular ontogeny and the location of the MLL breakpoint on leukemia initiation in vivo by transplanting t(4;11)-CRISPR/Cas9-edited cells into NSG mice (Figure S2G). No significant bias in overall engraftment potential was observed across the samples; however, marked differences were noted in the rates of engraftment driven by MA4-rearranged CD34+ cells (from 11% to 70%, Figure 2C). The frequency of Mi10A4+ and Mi12A4+ engraftment was higher when the translocation was generated in CB-CD34+ cells (16/28, 57%) as compared with FL-CD34+ cells (5/29, 18%). Furthermore, transplantation of these cells into secondary recipients resulted in human leukemic MA4+, NG2+, lymphoid-based (pro-B, CD19+CD10neg) and multi-organ (bone marrow, peripheral blood, spleen, and liver) grafts, except for Mi10A4 FL-derived cells, which failed to engraft after serial transplantation and consistently exhibited a non-leukemic phenotype (MA-, NG2-, multilineage) (Figure 2D). Of note, clinico-biological data from patients with MLLr B-ALL studied within the MLL recombinome consortium revealed a lower incidence (30% vs 49%) of centromeric breakpoints (i9-i10) in the MLL BCR among infant patients (<6 months) than among pediatric patients (>6 months)18 (Figure 2E). Collectively, our results show that genome-edited t(4;11)/MA4+ in human FL and CB CD34+ cells are sufficient to induce a transplantable MA4+ pro-B ALL that recapitulates key phenotypic features of MLLr iB-ALL, but the leukemogenic potential of MA4+ CD34+ cells appear to be influenced by the cellular ontogeny and the MLL breakpoint location within the MLL BCR.
We finally characterized the pro-B ALL generated from M\textsuperscript{10A4+}, M\textsuperscript{12A4+} CB-derived cells, and M\textsuperscript{12A4+} FL-derived cells in secondary mice. Recurrent mutations in \(K\)- and \(N\)-RAS were found, phenocopying those found in patients with primary iB-ALL \((n=4/9, 44\%, \text{ Figure } 2F)^{3,4}\), and confirming the leukemogenic potential of t(4;11)/MA4+ (Figure 2F). Differential methylation analysis revealed thousands of hyper- and hypomethylated sites (Figure S3A), and enrichment analyses of transcription factor binding sites and chromatin states indicated similarities in DNA methylation and chromatin patterns between leukemias from edited cells and primary MLLr iB-ALL (Figure S3B-C). Transcriptome and methylome analyses revealed that the pro-B ALL from edited cells clustered with primary MLLr iB-ALL from patients (Figure S3D-E). Overall, our study provides evidence that pro-B ALL generated \textit{in vivo} from MA4-edited CB- or FL-derived CD34+ cells recapitulate key molecular features of MLLr iB-ALL.

Our findings demonstrate that the origin of CD34+ cells does not affect the efficiency of t(4;11)/MA generation. Our human genome-editing t(4;11)/MA4+ B-ALL model recapitulates the \(HOX^{A9}\text{ and } HOX^{A10}\) transcriptomic signatures reported in t(4;11) B-ALL patients with telomeric-biased (M\textsuperscript{12A4+}) and centromeric-biased (M\textsuperscript{10A4+}) \(MLL\) breakpoint hotspots, respectively, regardless of the source (prenatal or neonatal) of the HSPCs. We confirmed that M\textsuperscript{10A4}, but not M\textsuperscript{12A4}, directly and specifically regulates the expression of \(HOXA9, HOXA10\) and \(MEIS1\) through binding to their regulatory regions. Although our \textit{ex vivo} model failed to replicate the upregulation of other previously reported genes, \(^{19}\) it establishes a link between \(HOX\) cluster gene expression and intronic distribution of \(MLL\) breakpoints in t(4;11)/MA4+ patients.

Our model also recapitulates the presence of recurrent mutations exclusively in \(K\)- and \(N\)-RAS, phenocopying those of patients with primary iB-ALL\(^{3,4}\), and confirming that t(4;11)/MA4+ is a leukemogenic driver. It is worth noting that targeting the \(MLL\) BCR of the centromeric region enhances \textit{in vitro} myeloid clonogenic replating in \(MLL\)-edited CD34+ HSPCs regardless of their cellular origin. These findings align with clinical and biological data\(^{18}\), and reveal an increased prevalence of AML among patients with MLL-r with a centromeric \(MLL\) breakpoint. However, all leukemias initiated in mice transplanted with either M\textsuperscript{10A4}-edited or M\textsuperscript{12A4}-edited CD34+ cells were proB-ALL, suggesting that the genomic localization of \(MLL\) breakpoint influences the resulting leukemia phenotype in cooperation with \textit{in vivo} occurring interactions in specific bone marrow niches\(^{20}\), secondary oncogenic hits\(^{21}\), and the nature of the cell-of-origin\(^{1}\). There remain some differences between human leukemias and engineered cells that warrant further exploration.

Human samples were accessed and processed following the institutional guidelines approved by our local Institutional Review Board (HCB/2013/8648).

Acknowledgments

We thank the MRC/Wellcome Trust Human Developmental Biology Resource (http://hdbr.org) for providing IRB-approved fetal material. We thank CERCA/Generalitat de Catalunya and Fundació Josep Carreras-Obra Social la Caixa for core support. Financial support for this work was obtained from the Spanish Ministry of Economy and Competitiveness (PID2022-142966OB-I00ID2019), “Heroes hasta la médula” initiative and ISCIII-RICORS within the Next Generation EU program.
(plan de recuperación, transformación y resiliencia) to PM and the Health Institute Carlos III (ISCIII/FEDER) PI20/00822 to CB, PI20/01837 to SR-P and PI21/01641 to RT-R. Asociación Española Contra el Cancer (AECC) (PRYGN211192BUEN) to CB, AECC-LABAE20049RODR to SR-P and the Fundación Uno entre Cienmil to CB. RT-R and OM were supported by investigator fellowships from the Spanish Association of Cancer Research (INVES211226MOLI). RM is supported by grants from the DFG (Ma 1876/12-1) and Wilhelm Sander foundation (2018.070.2).

Author contributions
CB, RT-R, TV-H, OM, PP, AM, VR, MV, SC, NG-G, SR-P, JCS, OQ-B ALS and JRT performed experiments and interpreted data. OW, MFF, AR, CM, RM, TAM, and PM supervised research and contributed key knowledge, techniques, and reagents. CB, RT-R and PM conceived the study and funded the research. All authors have read and agreed to publish the manuscript.

Declaration of interests
PM is founder of the spin-off OneChain Immunotherapeutics, which has no connection with the present research. The remaining authors declare no competing interests. TAM is a paid consultant for and shareholder in Dark Blue Therapeutics Ltd, a company that has no connection to the results in this paper.
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Legends to Figures

Figure 1. CRISPR-Cas9-induced t(4;11)/MA4 targeting either \textit{MLL}^{i10} (M^{i10}A4) or \textit{MLL}^{i12} (M^{i12}A4) in human FL and CB CD34+ HSPCs causes MA4-driven myeloid immortalization \textit{in vitro}. A) Scheme showing the location of the molecular regions targeted by sgMLL^{i10} or sgMLL^{i12} and sgAF4^{i3} sgRNAs (top) and the resulting t(4;11)/MA chromosomal translocation (bottom). Filled gray/orange boxes depict exons and the lines between boxes depict introns. B) Cartoon of the experimental design for \textit{in vitro} studies. C) Representative 60-day cell expansion of control and edited (M^{i10}A4 and M^{i12}A4) FL (left panel) and CB (right panel) CD34 myeloid progeny (CD45^+CD34^-CD19^-CD33^+). Two additional independent experiments for each cell of origin are shown in Figure S1A-B. D) Representative G-banding karyotype confirming the presence of chromosomal translocation after 42 days of culture. Enlarged images of the translocated chromosomes are also shown. E) \textit{MLL} split-apart iFISH quantification of \textit{MLL}-edited cells in control and edited cell cultures at the indicated time points, for FL (left panel) and CB (middle panel); right panels show representative images of \textit{MLLr} and \textit{MLL}-germline cells.

Figure 2. A centromeric location of the \textit{MLL} breakpoint within the \textit{MLL} BCR, but not the cellular ontogeny, determines the expression of HOX cluster/MEIS genes in \textit{MLL}-edited CD34+ cells. A) Heatmap showing clustering of \textit{MLL}-edited CD34+ HSPCs based on the location of the \textit{MLL} breakpoint (M^{i10}A4 n=6, blue versus M^{i12}A4 n=6, green) irrespective of the cellular ontogeny (FL (n=6, pink) versus CB (n=6, yellow)) based on significant differentially-expressed \textit{HOX} cluster genes (FDR <0.05) among the 26 genes included in the \textit{HOX} cluster. B) Representative ChIP-seq tracks at the MA4 target genes HOX9/10 and MEIS in FL (left panels) and CB (right panels) M^{i10}A4- and M^{i12}A4-edited cells. Binding of H3K79me3 and H3K27ac to HOX9/10 and MEIS was analyzed as a control. C) Leukemia incidence in primary and secondary recipient mice transplanted with FL- and CB-derived M^{i10}A4- and M^{i12}A4-edited HSPCs. Primary mice, n=57; secondary mice, n=42. D) Representative immunophenotype of a non-leukemic (green) and a leukemic (purple) mouse. Leukemic mice recapitulate many immunophenotypic features of primary MA4+ B-ALL such as pro-B-lymphoid (CD10^{neg})-biased engraftment (practically
depleted of myeloid graft) coupled to the expression of the \textit{MLL}-specific antigen NG2, and some degree of myeloid-lymphoid lineage infidelity/mixed phenotype (CD33+CD19+). Cells in gray are mouse cells. \textbf{E}) Clinical data from the international MLL Recombinome Taskforce reveals a higher frequency of telomeric (i11-i12) MLL breakpoints among patients with iB-ALL (0–6 months old); n=526 patients analyzed. \textbf{F}) Rates of N/K-RAS mutations determined by the Oncomine childhood leukemia mutational panel in leukemic mice (n=4/9, 44%) reproduce those reported in patients with primary iB-ALL.
**Figure 1**

**A**

![Diagram A](image)

**B**

**D0**

**Nucelofection**

FL- or CB-CD34+ cells

**In vitro proliferation**

**iFISH analysis**

**Clonogenic assays**

**Stemcell & Cytokines (IL3+FLT3L+SCF+IL7)**

**Molecular characterization**

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**C**

**FL**

![Graph FL A](image)

**CB**

![Graph CB A](image)

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**E**

**FL**

![Graph FL B](image)

**CB**

![Graph CB B](image)

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**D**

**FL:** MLL10AF4

![Graph FL C](image)

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**D**

**FL:** MLL12AF4

![Graph FL D](image)