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

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Abstract:

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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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Data Sharing: Reagents/protocols will be provided to other investigators upon contact with the corresponding author

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[1]. 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[1,2]. In fact, the MA4 fusion occurs in early hematopoietic stem/progenitor cells (HSPCs) and is a causal oncogenic driver in iB-ALL[3,4]. 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.[5] 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.[6,7] 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[8-12]. 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[13,14], 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*^{i10}) and telomeric (intron 12, *MLL*^{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, TableS1). Nucleofected FL and CB cells were cultured with cytokines to promote *MLL*-edited cell expansion[13] (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*^{i12}-rearranged cultures was consistently slower than *MLL*^{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-M^{i10}A4 *versus* telomeric-M^{i12}A4) irrespective of the cellular ontogeny (FL or CB) (Figure S2B). Specifically, the expression of *HOX* cluster genes, known to be involved in MA4-mediated pathogenesis[15,16], 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 M^{i10}A4-edited cells but not in M^{i12}A4-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 M^{i10}A4-edited FL and CB CD34$^+$ cells but not in M^{i12}A4-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 M^{i10}A4, but not M^{i12}A4, in regulating the expression of *HOXA9, HOXA10* and *MEIS1* through d
irect and specific binding to their regulatory regions. This recapitulates the HOXA^{low} transcriptomic signature reported in infant patients[3,15,17].

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 *MLL*^{i10}-edited cells displayed clonogenic capacity upon serial replating, regardless of their cellular ontogeny (Figures S2D-E). Notably, these results align with clinical data[18] 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 M^{i10}A4+ and M^{i12}A4+ 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$^+$CD10^{neg}) and multi-organ (bone marrow, peripheral blood, spleen, and liver) grafts, except for M^{i10}A4 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 $M^{i10}A4+$, $M^{i12}A4+$ CB-derived cells, and $M^{i12}A4+$ 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%, 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 *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 *HOXA*^{low} and *HOXA*^{hi} transcriptomic signatures reported in t(4;11) B-ALL patients with telomeric-biased ($M^{i12}A4^+$) and centromeric-biased ($M^{i10}A4^+$) *MLL* breakpoint hotspots, respectively, regardless of the source (prenatal or neonatal) of the HSPCs. We confirmed that $M^{i10}A4$, but not $M^{i12}A4$, directly and specifically regulates the expression of *HOXA9, HOXA10* and *MEIS1* through binding to their regulatory regions. Although our *ex vivo* model failed to replicate the upregulation of other previously reported genes,[19] it establishes a link between *HOXA* 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 *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 $M^{i10}A4$-edited or $M^{i12}A4$-edited CD34+ cells were proB-ALL, suggesting that the genomic localization of *MLL* breakpoint influences the resulting leukemia phenotype in cooperation with *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).

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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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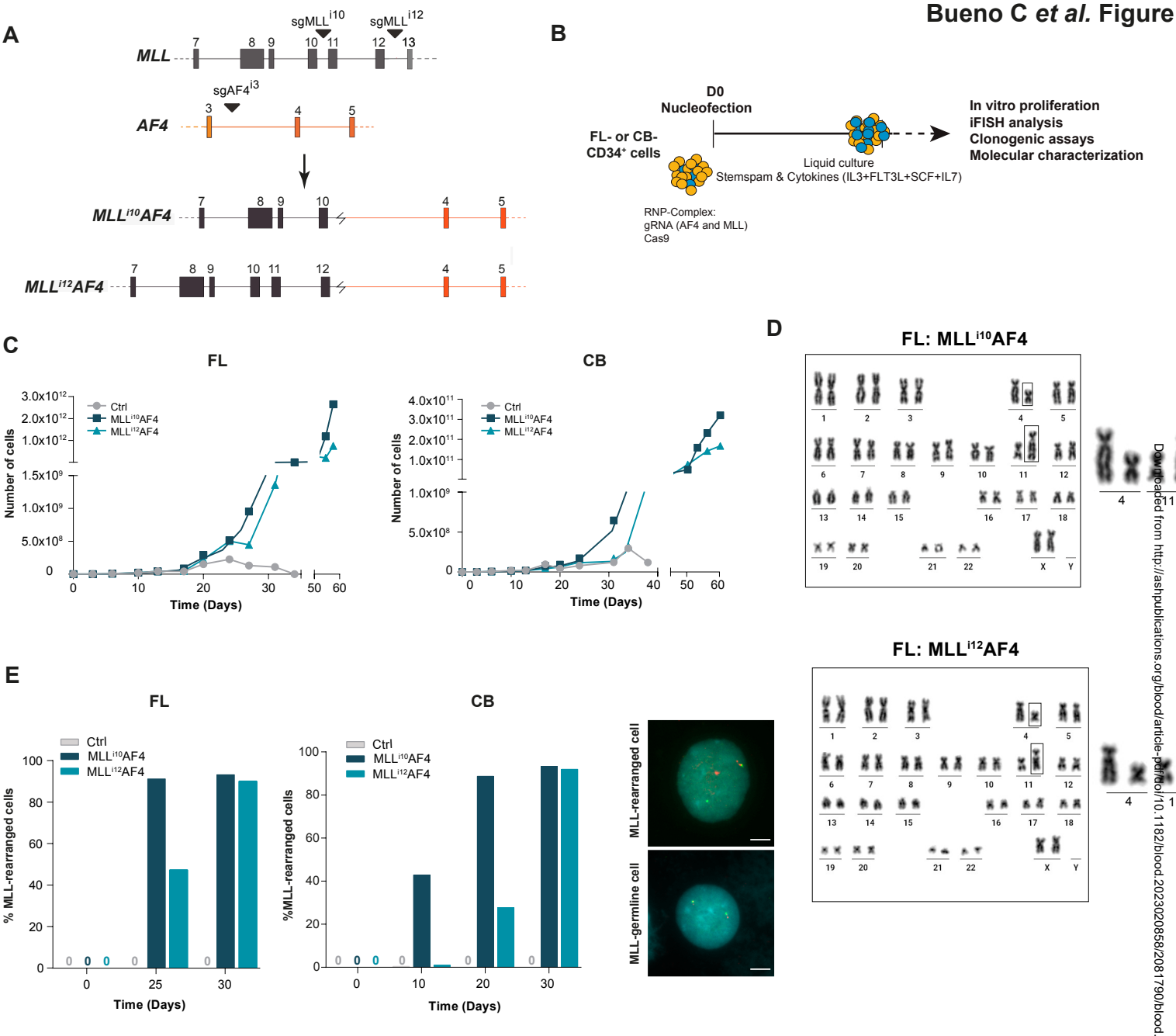
Legends to Figures

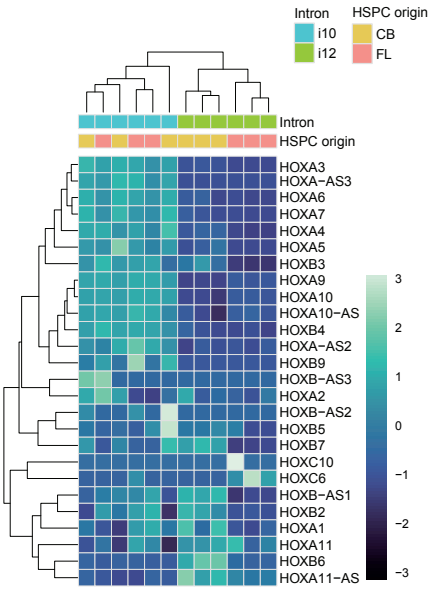
Figure 1. CRISPR-Cas9-induced t(4;11)/MA4 targeting either *MLL*^{i10} (M^{i10}A4) or *MLL*^{i12} (M^{i12}A4) in human FL and CB CD34^{+} HSPCs causes MA4-driven myeloid immortalization *in vitro*. A) Scheme showing the location of the molecular regions targeted by sgMLL^{i10} or sgMLL^{i12} and sgAF4^{i3} 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 *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) *MLL* split-apart iFISH quantification of *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 *MLL*r and *MLL*-germline cells.

Figure 2. A centromeric location of the *MLL* breakpoint within the *MLL* BCR, but not the cellular ontogeny, determines the expression of HOX cluster/MEIS genes in *MLL*-edited CD34^{+} cells. A) Heatmap showing clustering of *MLL*-edited CD34^{+} HSPCs based on the location of the *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 *HOX* cluster genes (FDR <0.05) among the 26 genes included in the *HOX* cluster. B) Representative ChIP-seq tracks at the MA4 target genes *HOXA9/A10* 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 *HOXA9/A10* 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 *MLL*-specific antigen NG2, and some degree of myeloid-lymphoid lineage infidelity/mixed phenotype ($CD33^{+}CD19^{+}$). Cells in gray are mouse cells. 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. 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.



A



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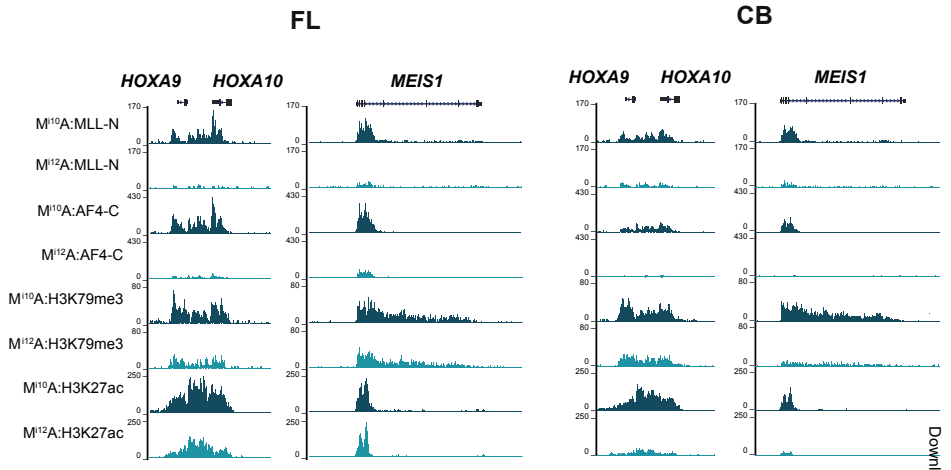
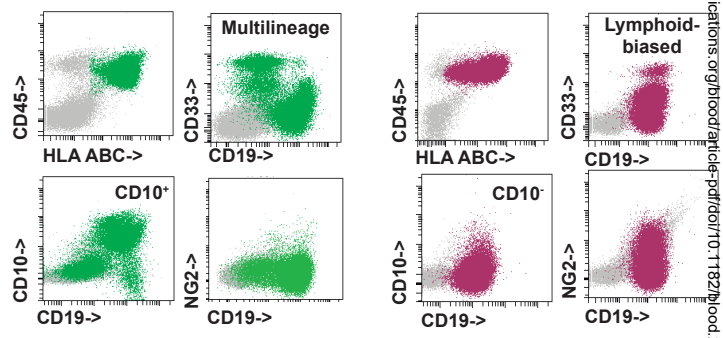


C

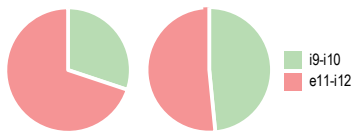
		Primografts		Secondary transplants	
		Engrafted mice	PCR^{pos}	Engrafted mice	PCR^{pos}
CB	M^{i10}A4	15/15 100%	9/15 70%	18/18 100%	18/18 100%
	M^{i12}A4	13/15 86%	7/13 54%	14/14 100%	14/14 100%
FL	M^{i10}A4	18/24 75%	2/18 11%	0/4 0%	0/4 0%
	M^{i12}A4	11/15 78%	3/11 27%	6/6 100%	6/6 100%

D



E

Age (months)	Centromeric (i9-i10)	Telomeric (e11-i12)	Total cases
0-6	98 (30%)	227 (70%)	325
6-24	98 (49%)	103 (51%)	201



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