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Citation for published version:

Digital Object Identifier (DOI):
10.3324/haematol.2018.191718

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Haematologica

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The fetal liver lymphoid-primed multipotent progenitor provides the prerequisites for the initiation of t(4;11) MLL-AF4 infant leukemia

**Running title:** The cell-of-origin of MLL-AF4+ infant leukemia

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**Conflicts of interests**

The authors have no conflicts of interest to disclose.

**Grant Support**

Bloodwise, Kay Kendall Leukaemia Fund, MRC, Cancer Research UK

Word count: 1454
t(4;11) MLL-AF4 pro-B acute lymphoblastic leukemia (ALL) is an aggressive hematological malignancy that accounts for 50-85% of infant ALL cases. Retrospective analysis of Guthrie cards and twin concordance studies both confirmed the pre-natal origin of this disease. Patients show an accumulation of immature pro-B cells in the bone marrow, followed by a rapid and uncontrolled proliferation of leukemia blasts that hijack the immune system and invade peripheral organs such as the spleen, liver and central nervous system. The chromosomal translocation results in the fusion of the N-terminal part of MLL with almost the entire AF4 gene, which disrupts the epigenetic signature of hematopoietic cells. This induces a stem cell-like expression signature (e.g. HOXA cluster, MEIS1, RUNX1) as well as a pro-survival and proliferation phenotype (characterized by the upregulation of BCL2, MCL1, CDK6).

The molecular signature at diagnosis has been well characterized, but information on the initial changes during the first stages of leukemogenesis is lacking due to the challenge posed by the pre-natal origin of the disease. We recently described a pre-leukemia model of t(4;11) MLL-AF4 infant leukemia, which uses an Mll-AF4 invertor line and a VE-Cadherin-driven Cre recombinase to target the expression of Mll-AF4 to all definitive hematopoietic cells formed during embryonic development. While this model does not progress to the same rapid, acute leukemia phenotype observed in human patients, possibly due to species differences, it provides unique access to the prenatal pre-leukemic state in vivo. Mll-AF4 expression was shown to lead to increased engraftment and self-renewal potential of E14 fetal liver (FL) cells, as well as a high B lymphoid clonogenic potential; however, the precise contribution from individual cell types was not addressed in detail.

Here, we separated the hematopoietic compartment into three stem/progenitor fractions, HSC/MPPs, LMPPs and LK/CLPs, with a sorting strategy adapted to the fetal context (Figure S1A) and used transplantation assays and gene expression analysis to further characterize the cell-of-origin of t(4;11) MLL-AF4 pro-B ALL. Extensive details on the material and methods can be found in the supplementary section. Analysis of cell cycle distribution showed that the HSC/MPP population was more highly represented in the G0-G1 phase compared to LMPP and LK/CLP (Figure S1B), and less in the G2/M phase (Figure S1C). Mll-AF4 did not alter the cell cycle distribution of HSC/MPPs, LMPPs or LK/CLPs, suggesting that proliferation is not hijacked during early stages of leukemogenesis.
All three fractions were transplanted to assess their engraftment, self-renewal and differentiation potentials. Mll-AF4 did not affect the engraftment of HSC/MPPs or LK/CLPs, but led to a significantly higher engraftment of the LMPP fraction (Figure 1A-C). We previously found that HSC/MPPs and LMPPs from E14 FL could form B-lymphoid colonies with a pro-B phenotype when Mll-AF4 is expressed. We therefore assessed the lineage output in the peripheral blood of the primary recipients. While there was no difference in T (CD3+) and mature B cell (B220+CD19+IgM+) production, LMPPs had a lower myeloid (CD11b+Gr1+) and higher immature B cell (B220+CD19+IgM-) output compared with HSC/MPPs (Figure 1D). This skewing was, however, independent of Mll-AF4 expression and therefore represents an intrinsic property of LMPPs. Mll-AF4+ LMPP primary recipients had the highest white blood cell count one month after transplant, suggesting a faster contribution to the hematopoietic system (Figure 1E); however, this difference diminished over time. The expression of Mll-AF4 is significantly higher in E14 fetal liver LMPPs compared to HSC/MPPs and LK/CLPs, which may offer an explanation for their enhanced engraftment in primary recipients (Figure 1F, left set of graphs).

We then assessed the self-renewal potential of HSC/MPPs and LMPPs in secondary transplantations. Mll-AF4 expression increased the repopulation of secondary recipients with HSC/MPPs compared with the Mll-AF4- control (Figure 1G), although engraftment levels were not significantly different from those observed with Mll-AF4+ HSC/MPPs in primary recipients (Figure S1D). Mll-AF4+ LMPPs, on the other hand, only showed limited self-renewal (Figure 1G), suggesting that Mll-AF4 can only enhance self-renewal in cells that already possess this property. Furthermore, we detected an upregulation of Mll-AF4 expression following transplantation in HSC/MPPs sorted from primary recipients, while it remained unchanged in LMPPs, which may also contribute to the higher self-renewal potential of Mll-AF4+ HSC/MPPs (Figure 1F, right set of graphs). Both populations displayed skewing towards the B lineage in the presence of Mll-AF4 in secondary recipients, which, in the case of LMPPs, resulted in an almost entirely B lymphoid-biased output (Figure 1H). The white blood cell count was significantly higher in Mll-AF4+ HSC/MPP secondary recipients from two months after transplantation (Figure 1I).

We did a post-mortem analysis of HSC/MPP secondary recipients and LMPP primary recipients (6-15 months old). As shown above, hematopoietic progenitors such as LMPPs, can only engraft primary recipients, whereas HSCs can serially
engraft. Therefore, we compared LMPP primary recipients and HSC/MPP secondary recipients to assess differences in hematopoietic output established by HSCs and LMPPs. Mll-AF4+ HSC/MPPs and LMPPs showed a significantly higher engraftment in the bone marrow and liver compared to Mll-AF4- HSC/MPPs (Figure 1J). Furthermore, LMPP primary recipients had more donor cells in the spleen compared to Mll-AF4+ HSC/MPP recipients. While there were no significant differences in the stem/progenitor compartment in the bone marrow (Figure 1K), Mll-AF4+ LMPP primary recipients displayed a trend towards more pro-B cells in the spleen compared to Mll-AF4+ HSC/MPP secondary recipients (Figure 1L), which resulted in a higher proportion of mature B220+CD19+IgM+ cells (Figure 1M).

To explain the cell type-specific effects, we assessed the expression of 14 genes associated with t(4;11) MLL-AF4 pro-B ALL and, more specifically, linked to HSC signature, B-lymphoid differentiation, cell division and pro-survival phenotypes: \(Flt3\), \(Meis1\), \(Hoxa9\), \(Hmga2\), \(Lmo2\), \(Runx1\), \(Cdk6\), \(Il7r\), \(Pax5\), \(Ikaros\), \(E2a\), \(Bcl-2\), \(Mcl1\) and \(Twist1\) (Figure 2A). \(Flt3\) is upregulated in LMPPs regardless of Mll-AF4 status (Figure 2B), while \(Meis1\) is upregulated specifically in response to Mll-AF4 expression in LMPPs (Figure 2C). The expression of \(Hoxa9\) in Mll-AF4+ LMPPs is significantly higher compared with Mll-AF4+ HSC/MPPs and/or Mll-AF4+ LK/CLPs (Figure 2D). \(Hmga2\), \(Lmo2\) and \(Runx1\) expression in Mll-AF4+ LMPPs is significantly higher compared with Mll-AF4+ HSC/MPPs and/or Mll-AF4+ LK/CLPs (Figure 2E-G). \(Cdk6\) expression was higher in LK/CLPs, and decreased in HSC/MPPs upon Mll-AF4 expression (Figure 2H). This can partly explain the enhanced self-renewal of Mll-AF4+ HSC/MPPs compared to Mll-AF4- HSC/MPPs (Figure 1G). The expression of \(IL7r\) and \(Pax5\) was generally higher in LMPPs, but was not significantly affected by Mll-AF4 expression, although there was a clear trend (Figure 2I,J), whereas \(Ikaros\) and \(E2a\) were strongly upregulated in Mll-AF4+ LMPPs compared to Mll-AF4- LMPPs and Mll-AF4+ HSC/MPPs and LK/CLPs (Figure 2K,L). This likely explains the strong B-lymphoid bias observed in the transplant recipients (Figure 1H). We observed a significant upregulation of \(Bcl-2\) in Mll-AF4+ LMPPs compared to Mll-AF4- LMPPs (Figure 2M), which is a direct transcriptional target of MLL-AF4.\(^7\) \(Mcl1\) expression was relatively stable (Figure 2N), but \(Twist1\) was upregulated in Mll-AF4+ LMPP compared to HSC/MPP and LK/CLP Mll-AF4+ cells (Figure 2O). We assessed the expression of \(Flt3\), \(Meis1\), \(Hoxa9\), \(E2a\) and \(Bcl-2\) in sorted Mll-AF4+ HSC/MPPs, LMPPs and LK/CLPs from primary recipients to measure expression changes induced
by transplantation stress and a change in microenvironment: FL versus bone marrow (Figure 2B,C,D,L,M). The relative expression pattern amongst the three populations was similar in freshly sorted FL cells and sorted cells from primary recipients. However, there is a general upregulation of Flt3, Meis1, Hoxa9 and E2a in cells from primary recipients (Figure 2B,C,D,L), which may explain the shorter disease latency following transplantation observed previously. The strong upregulation of E2a also likely explains the strong B lymphoid bias observed in LMPP recipients (Figure 1H). This study suggests that the FL LMPP sets the stage for the transformation process of t(4;11) MLL-AF4 infant pro-B ALL through the higher expression of Flt3, Hoxa9, Lmo2, Runx1, Il7r, Pax5, E2a and Twist1 (Figure 2P). The activation of Mll-AF4 increases the expression of E2a, Ikaros, Meis1 and Bcl-2, leading to a strong B lymphoid bias and a survival advantage. This study is a step forward in understanding the molecular mechanisms of infant leukemogenesis, and also further supports our previous proposition that the FL LMPP is the cell-of-origin of t(4;11) Mll-AF4 pro-B ALL.
Acknowledgements

This study was supported by a Bloodwise Bennett Senior Fellowship (K.O.) and a grant from the Kay Kendall Leukaemia Fund and Cancer Research UK (K.O.). The authors would like to thank Fiona Rossi and Claire Cryer from the SCRM FACS Facility for cell sorting services and flow cytometry advice and would like to acknowledge the support of Andrew Dyer, Hollie McGrath, Linda Dunn-Campbell and John Agnew from the SCRM Animal Facility in animal experimentation. Core facilities at the Edinburgh MRC Centre for Regenerative Medicine by centre grant MR/K017047/1.
Authorship Contributions

C.M. performed and designed experiments, analyzed the results and wrote the manuscript. K.O. supervised the study and wrote the manuscript.
References

Figure legends

Figure 1. In the presence of Mll-AF4, E14 FL LMPPs display higher engraftment potential and a B-lymphoid bias. (A–C) Primary transplant of 1000 HSC/MPPs (A), 750 LMPP (B) and 1000 LK/CLP (C). Total donor chimerism in peripheral blood is shown and dotted line represents 5% threshold for considering mice as being repopulated. Repopulated mice/total injected shown next to the curve. (D) Donor chimerism in individual lineages of primary recipients 4 months after transplant. (E) White blood cell count in the peripheral blood of primary recipients. (F) Quantitative PCR of Mll-AF4 in fresh fetal liver HSC/MPP, LMPP and LK/CLP and sorted cells from primary recipients. (G) Secondary transplant of HSC/MPP and LMPP-derived bone marrow cells from primary recipients. (H) Donor chimerism in individual lineages of secondary recipients 4 months after transplant. (I) White blood cell count in the peripheral blood of secondary recipients. (J) Donor chimerism in the peripheral blood, bone marrow, spleen and liver at end of study. (K) Donor-derived HSCs, LMPPs, CLP and LK in the bone marrow at end of study. (L) Donor-derived pre-pro-B and pro-B cells in the bone marrow and spleen at end of study. (M) Donor chimerism in individual lineages at end of study. A non-parametric Mann-Whitney test was used to compare datasets with a significance cut-off of p < 0.05 (*), p < 0.01 (**) or p < 0.001 (***) or p < 0.0001 (****).

Figure 2. The LMPP population displays an MLL-AF4 gene expression signature. (A) Quantitative PCR strategy in fresh fetal liver cells (n = 6-7) and cells derived from primary recipients (n = 3). Quantitative PCR of (B) Flt3, (C) Meis1, (D) Hoxa9, (E) Hmga2, (F) Lmo2, (G) Runx1 (H) Cdk6, (I) Il7r, (J) Pax5, (K) Ikaros, (L) E2a, (M) Bcl2, (N) Mcl1, (O) Twist1. (P) Early stages of t(4;11) MLL-AF4 infant leukemia based on the pre-leukemia mouse model. A non-parametric Mann-Whitney test was used to compare datasets with a significance cut-off of p < 0.05 (*), p < 0.01 (**) or p < 0.001 (***) or p < 0.0001 (****).
Quantitative PCR of HSC/MPP, LMPP and LK/CLP
E14 FL Mll-AF4-VEC-Cre+ and Mll-AF4+VEC-Cre+, Primary recipients Mll-AF4+VEC-Cre+

A
Stem-cell signature
Fit3
Meis1
Hoxa9
Hmg2a
Lmo2
Runx1
Cell division
Cdk6
B-lymphoid differentiation
IL7R
Pax5
Ikaros
E2A
Pro-survival genes
Bcl-2
Mcl1
Twist1

B
Fit3
C
Meis1
D
Hoxa9

E
Hmg2a
F
Lmo2
G
Runx1
H
Cdk6

I
Il7r
J
Pax5
K
Ikaros
L
E2A

M
Bcl-2
N
Mcl1
O
Twist1

P Early stages of t(4;11) MLL-AF4 infant pro-B ALL (fetal liver)

HSC/MPP
LMPP
LK/CLP

↑ Mll-AF4
↓ Self-renewal (↓ Cdk6)

↑ Mll-AF4

Stem-cell signature (↑ Meis1)
Pro-survival genes (↑ Bcl-2)
B-lymphoid bias (↑ E2a ↑ Ikaros)
Supplementary Material and Methods

Mice
The animal work was done under regulation of the UK Home Office. Males and females from the Mll-AF4\(^1\) and the VEC-Cre\(^2\) line were mated to obtain Mll-AF4-expressing embryos. The day of the plug was counted as day 0 of embryonic development.

Cell sorting of HSC/MPP, LMPP and LK/CLP populations
The fetal liver was dissected and dissociated in Flow Cytometry Staining Buffer (ThermoFisher Cat# 00-4222-26) using a 21Gx15mm needle attached to a syringe (BD Microlance Cat# 10472204-X, BD Cat# 3000185). Cells were stained using the following antibody mix in Flow Cytometry Staining Buffer: APC anti-mouse CD3\(\varepsilon\) antibody (clone I45-2C11, Biolegend Cat# 100312), APC anti-mouse TER-119 antibody (clone TER119, Biolegend Cat# 116212), APC anti-mouse F4/80 antibody (clone BM8, Biolegend Cat# 123116), APC anti-mouse Nk1.1 antibody (clone PK136, Biolegend Cat# 108709), APC anti-mouse Ly-6G/Ly-6C (Gr-1) antibody (clone RB6-8C5, Biolegend Cat# 108412), PE/Cy7 anti-mouse/human CD45R/B220 antibody (clone RA3-6B2, Biolegend Cat# 103222), PE/Cy7 anti-mouse CD19 antibody (clone 6D5, Biolegend Cat# 115520), APC-eFluor 780 CD117 (cKit) antibody (clone 2B8, ThermoFisher Cat# 47-1171-80), Alexa Fluor® 700 anti-mouse CD45 antibody (clone 30-F11, Biolegend Cat# 103128), Pacific Blue™ anti-mouse Ly-6A/E (Sca-1) antibody (Clone E13-161.7, Biolegend Cat# 122519), PE anti-mouse CD127 antibody (clone A7T34, ThermoFisher Cat# 12-1271-82), biotin anti-mouse CD135 (Flt3) antibody (clone A2F10, ThermoFisher Cat# 13-1351-81). Cells were stained for 20 minutes on ice and washed once with Flow Cytometry Staining Buffer. Cells were then resuspended in diluted Qdot 655 Streptavidin Conjugate (ThermoFisher Cat# Q10123MP) and incubated for 20 minutes on ice. Cells were washed once and resuspended in diluted SYTOX™ Green Nucleic Acid Stain (ThermoFisher Cat# S7020) to exclude dead cells. Sorting was done on a BD FACSARia™ II (BD Biosciences).
Transplantation of CD45.1/2 mice with HSC/MPP, LMPP and LK/CLP

On the day of transplant, recipient mice (CD45.1/2) received two doses of 4.6 Gy at a 3 hours interval. Donor cells (CD45.2/2) were injected through the tail vein along with bone marrow helper cells (CD45.1/1). For HSC/MPP transplants, we used 100 000 helper cells and for LMPP and LK/CLP, we used 20 000 helper cells. Mice were administered antibiotics after transplantation through their drinking water (0.1 mg/mL enrofloxacin, 10% Baytril solution from Bayer). For secondary transplants, the number of bone marrow cells transplanted was adjusted according to the repopulation in primary recipient (85% repopulation in primary recipient, 2 x 10^6 total bone marrow cells injected). Mice were bled on a monthly basis, and blood counts were measured on a Celltac MEK-6500K (Nihon Kohden). Red blood cell lysis was achieved with BD Pharm Lyse™ lysing solution according to the manufacturer’s instructions (BD Biosciences Cat# 555899). Cells were stained in Flow Cytometry Staining Buffer using the following antibodies: FITC CD45.2 antibody, (clone 104, ThermoFisher Cat# 11-0454-81), APC-eFluor 780 CD45.1 monoclonal antibody (clone A20, ThermoFisher Cat# 47-0453-80), eFluor450 CD11b monoclonal antibody (clone M1/70, ThermoFisher Cat# 48-0112-80), Alexa Fluor® 700 Ly-6G/Ly-6C (Gr-1) antibody (clone RB6-8C5, Biolegend Cat# 108422), PE/Cy7 CD45R/B220 antibody (clone RA3-6B2, Biolegend, Cat# 103222), Brilliant Violet 605™ CD19 antibody (clone 6D5, Biolegend Cat# 115539), APC mouse IgM monoclonal antibody (clone II/41, ThermoFisher Cat# 17-5790-82), PE CD3e (clone 145-2C11, Biolegend Cat# 100308). For sorting/analysis of hematopoietic stem and progenitor cells in organs and analysis of B cell populations in primary and secondary recipients, we used the following antibodies: the APC lineage cocktail from the sorted E14 FL cells, FITC CD45.2 antibody, (clone 104, ThermoFisher Cat# 11-0454-81), APC-eFluor 780 CD45.1 monoclonal antibody (clone A20, ThermoFisher Cat# 47-0453-80), Brilliant Violet 421™ CD117 (c-Kit) antibody (clone 2B8, Biolegend Cat# 105827), APC-eFluor 780 CD117 antibody (clone 2B8, ThermoFisher 47-1171-82), PE/Cy7 Ly6A/E (Sca-1) antibody (clone E13-161.7, Biolegend Cat#122513), PB Sca1 antibody (clone E13-161.7, Biolegend, Cat# 47-1171-82), PerCP/Cy5.5 CD34 antibody (clone HM34, Biolegend, Cat# 128607), PE CD135 antibody (clone A2F1, Biolegend, Cat# 135306), biotin anti-mouse CD135 (Flt3) antibody (clone A2F10, ThermoFisher Cat# 13-1351-81), Alexa Fluor® 700 CD48 antibody (clone HM48-1, Biolegend, Cat# 103425), PE/Cy7 CD150 antibody (clone TC15-12F12.2, Biolegend, Cat# 115914), Alexa
Fluor® 700 CD45R/B220 antibody (clone RA3-6B2, Biolegend, Cat# 103232), PE/Cy7 CD19 antibody (clone 6D5, Biolegend, Cat# 115520), PerCP CD43 antibody (clone 1B11, Biolegend, Cat# 121222), Brilliant Violet 421™ CD24 antibody (clone M1/69, Biolegend, Cat# 101825), APC CD127 antibody (clone A7R34, Biolegend, Cat# 135011). Cells were incubated on ice for 20 minutes, washed twice with Flow Cytometry Staining Buffer and resuspended in diluted SYTOX AADvanced (ThermoFisher, Cat# S10274) to exclude dead cells. Data was acquired on a BD LSRFortessa™ (BD Biosciences). For end of study analysis, cell types were identified as follows: HSCs (LSK CD34+/ - FLT3- CD150+ CD48-), LMPPs (LSK CD34+/ - FLT3+), CLP (Lin- ckitlow Sca1low IL7R+ FLT3+), LK (Lin- ckit+ Sca1-), pre-pro-B (CD45.2+ ckit- CD43+ CD24low B220+ CD19-) and pro-B (CD45.2+ ckit+ CD43+ CD24+ B220+ CD19+).

Cell Cycle
Sorted HSC/MPP, LMPP and LK/CLP cells were collected in Flow Cytometry Staining Buffer and an equivalent volume of 5 μg/mL DAPI 1% IGEPAL (Sigma-Aldrich D9542 and CA-630) solution was added. Cells were incubated at room temperature for 1 minute, in the dark. Data was acquired on a BD LSRFortessa™ (BD Biosciences).

RNA extraction, reverse transcription and quantitative PCR
RNA extraction and reverse transcription were performed using the RNeasy Micro Kit (QIAGEN Cat# 74004) and iScript Ready-to-Use cDNA Supermix (Bio-Rad Laboratories Ltd Cat# 1708841) according to the manufacturer’s instructions. Primer sequences are: Flt3.F/R (gccagttagccgccttctgctca/cgcagcctgcgtgctac), Meis1.F/R (cctcagttgggcttctgcttctgca), Cdk6.F/R (cctcagttgggcttctgcttctgca), McI1.F/R (cctcagttgggcttctgcttctgca), Twist1.F/R (cctcagttgggcttctgcttctgca), RunX1.F/R (cctcagttgggcttctgcttctgca), Hoxa9.F/R (cctcagttgggcttctgcttctgca), Hmga2.F/R (cctcagttgggcttctgcttctgca), Bcl2.F/R (cctcagttgggcttctgcttctgca), Lmo2.F/R (cctcagttgggcttctgcttctgca), Ikaros.F/R (cctcagttgggcttctgcttctgca), Il7r.F/R
(cgaaactccagaaccccaaga/aatggtgacacttggaagac), \(E2a.F/R\)
(aagaggacaagaaggacctgaa/ttatggccatacgctctc), \(Pax5.F/R\)
(acciaccaggacagcatgg/gcggacacatctgggagtg), \(Mll-AF4\) \(F/R\)
(agtggcatgtaggggatc/atggctcagctgtactaggc) and \(beta\)-actin \(F/R\)
(ttcgtctactgtcca/gtccgcctagaagcacttg) Quantitative PCR was carried out with Brilliant III Ultra-Fast SYBR QPCR (Agilent Technologies Cat#600883) according to the manufacturer’s instructions. Data was acquired on a QuantStudio™ 7 Flex Real-Time PCR System (ThermoFisher).

**Data analysis, statistics and graphs**

Analysis of flow cytometry data was performed with FlowJo (version 10) and graphs were generated with GraphPad Prism (version 6). Statistical analysis was performed with GraphPad using a non-parametric t test (Mann-Whitney) with a bi-lateral p-value. Data are presented as Mean ± SEM.
References for the supplementary


Figure S1. Sorting strategy and cell cycle analysis of E14FL HSC/MPP, LMPP and LK/CLP cells (A) Sorting strategy of E14 FL HSC/MPP (Lin- B220- CD19- CD45+ ckit+ Sca1high IL7R- Flt3-), LMPP (Lin- B220- CD19- CD45+ ckit+ Sca1high Flt3+) and LK/CLP (Lin- B220- CD19- CD45+ ckit+ Sca1low/-). (B,C) Cell cycle analysis (n = 5-7). HSC – hematopoietic stem cell, MPP – multipotent progenitor, LMPP – lymphoid-primed multipotent progenitor, LK – Lin- ckit+, CLP – common lymphoid progenitor. (D) Repopulation of Mll-AF4+ VEC-Cre+ E14 FL HSC/MPPs in primary and secondary recipients.