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MLL-AF9 expression levels do not instruct lineage fate

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Author contributions

E.K. and J.J.S. conceived and designed the study; E.K., M.C., A.B-V., B.d.B., D.P.M., G.H., J.J.S performed experiments, analyzed, and interpreted data, performed statistical analysis, and drafted the article; E.K. and J.J.S. wrote the paper which was reviewed by all authors. All authors gave final approval of the submitted manuscript.

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Letter To the editor

The t(9;11) translocation results in expression of the MLL-AF9 oncofusion protein which can give rise to either acute myeloid leukemia (AML) or B-cell acute lymphoblastic leukemia (B-ALL) in pediatric patients.[1,2] A recent study suggested that oncogene expression levels instruct lineage choice and that B-ALL patient treatment with menin inhibition may be complicated by lineage switching promoting AML relapse.[3] By utilizing an exogenous lentiviral approach we were able to specifically address whether or not MLL-AF9 oncofusion protein levels causally specify lineage fate. Additionally, we show that the previously reported findings most likely are confounded by the lineage inherent epigenetic landscape determining MLL1 expression, which subsequently dictates oncogene expression levels.

Previously, various studies have addressed the question of whether cell intrinsic or cell extrinsic mechanisms would underly lineage choice in *MLL-AF9*-driven leukemic transformation. Clearly, cell extrinsic factors play an important role, as cord blood (CB) $CD34^+$ cells lentivirally transduced with *MLL-AF9* predominantly give rise to B-ALL when transplanted into lymphoid-biased NSG mouse models, while by transplantation into more myeloid permissive humanized mice expressing human cytokines both AML and B-ALL phenotypes were observed.[4–7]

Oncogene dosage impacts on the cell of origin from which *MLL-AF9*-induced leukemia can arise. While initial retro/lentiviral overexpression models showed that the *MLL-AF9* oncogene can efficiently transform committed GMPs into leukemic GMPs (L-GMPs),[8] it was later shown that when *MLL-AF9* was expressed under its own endogenous promoter only the most immature $Lin^-Sca1^+/cKit^+$ progenitors were susceptible to *MLL-AF9*-induced transformation.[9] Clearly, the oncogene expression levels dictate whether or not *MLL-AF9* can re-install a self-renewal and oncogenic transcriptional program in cells that have already committed to the myeloid lineage.

Cell of origin dynamics have also been observed for *MLL1* gene itself. Unlike in committed cells, *MLL1* has been found to be essential for the survival and self-renewal of hematopoietic stem cells.[10] However, the comparison of MLL/MLLr gene expression and subsequent effects is conflicted by the fact that the binding targets have been found to be different.[11]

Rare cases of conversion of the lymphoid and myeloid leukemic cell lineage during the course of the disease or during relapse have been reported in literature. According to published data, lineage switching is almost exclusively observed in pediatric patients and it occurs most frequently from the lymphoid to the myeloid

lineage.[12,13] In adults, *MLL-AF9* almost exclusively results in AML and not B-ALL, and lineage switching is rarely seen. Several hypotheses have been proposed to explain the molecular basis of the lineage switch, but the actual mechanisms remain to be discovered. Possible explanations are the emergence of new therapy-related clones or the emergence of therapy-insensitive clones of a different lineage already present at low frequencies at initial diagnosis. Another explanation might be the reprogramming of a multipotent leukemic stem cell that in response to intrinsic or environmental cues can change the lineage of its progeny.[14] In *MLL-AF4* pediatric leukemia, it was shown that lymphoid to myeloid lineage switching is driven by disrupted epigenetic regulation.[15] Although these complete lineage switches might occur infrequently, it is relevant to evaluate whether the leukemic blasts originate from the same leukemic clone or whether new clones emerge as a consequence of the treatment.

We first addressed this question in an *MLL-AF4* patient with a mixed leukemia phenotype presenting with both CD19$^+$ lymphoid clones as well as CD33$^+$ myeloid clones (Figure 1a). Both clones were sorted and cultured under myeloid-restricted MS5 coculture conditions, or under lymphoid-permissive MS5 coculture conditions under which both myeloid and lymphoid cells can propagate. These data demonstrated that CD19$^+$ clones retain myeloid potential whereas CD33$^+$ clones failed to produce lymphoid output (Figure 1a). Morphologically (Figure 1a) as well as transcriptionally (Figure 1b-e) CD19$^+$-sorted cells cultured under myeloid conditions indeed switched to a myeloid phenotype. When analyzing expression of typical *MLL*r target genes such as HOXA9, FLT3 and PBX we noted that expression levels appeared to be somewhat higher in lymphoid clones (Figure 1e), potentially indicating that MLL-AF4 is higher expressed in those cells. This patient sample was derived from the OncoLifeS data-biobank for Oncology (Dutch Trial Register: NL7839) which entails written informed consent and approval of the study protocol by the local Institutional Review Board (UMCG METc2010/109) in accordance with the Declaration of Helsinki.

Similarly to the *MLL-AF4* patient sample, in CB lentiviral transduction models we could show that cells that were initially transformed along the B-lymphoid lineage retained myeloid potential when cultured under myeloid-restricted culture conditions (Supplemental Figure 1a-b). When CD19$^+$ lymphoid-transformed cells were cultured under lymphoid permissive conditions for 1-3 weeks, cells retained a lymphoid phenotype, but when cells were grown under myeloid restricted conditions, cells gradually lost CD19 expression and became positive for CD33, CD11b, CD14 and

CD15 (Supplemental Figure 1a-b). The growth rate of cells under both culture conditions remained similar (Supplemental Figure 1c). LM-PCR analysis indicated that the integration site in the initial CD19$^+$ lymphoid transformed cells was retained in the myeloid cells, indicating that the lineage shift did not occur because of clonal drift (Supplemental Figure 1d).

A recent report suggested that MLL oncoprotein levels would influence leukemia lineage identities.[3] Whether or not higher MLL-AF9 levels are indeed causally related to transformation along the lymphoid lineage remained to be proven functionally. The use of lentiviral models where expression of the oncogene is driven by an artificial promoter that is independent of lineage type provides a unique opportunity to study whether causal relationships exist between *MLL-AF9* oncogene dosage and lineage fate. Here, we employed a lentiviral exogenous expression model in CB CD34$^+$ cells which allowed the development of either AML or B-ALL *in vitro* and *in vivo* upon transplantation in myeloid permissive MISTRG mice. *MLL-AF9* expression is driven by the UBC promoter, and our vector includes an internal ribosomal entry site (IRES) followed by the EGFP reporter gene. In these assays, we usually observe the outgrowth of distinct clones marked with different levels of EGFP corresponding to different *MLL-AF9* transcript levels as determined by qPCR (see below).

Under *in vitro* lymphoid-permissive MS5 co-cultures that allow outgrowth of B lymphoid and myeloid cells we observed that *MLL-AF9*-expressing cells could give rise to both AML and B-ALL clones marked by CD33 and CD19, respectively (Figure 2a). Measuring the mean fluorescent intensity (MFI) of the EGFP signal of the separate populations revealed that B-ALL clones consistently had lower MFI levels compared to the AMLs (Figure 2a, b). *In vivo* data upon transplantation in MISTRG mice showed similar results in bone marrow, spleen and liver engrafted cells (Figure 2c-d). Sorting of the separate clones from *in vitro* and *in vivo* experiments and determination of the *MLL-AF9* oncogene mRNA levels confirmed that EGFP MFI was a good indicator of the oncogene transcript levels (Supplemental Figure 2a-b; Figure 2e). When the same analysis was repeated for the empty vector (EV)-mCherry MFI, which was co-transduced as an internal control, no difference was observed between the B-ALLs and AMLs (Supplemental Figure 2c-e). Collectively, our results indicate that higher levels of MLL-AF9 fusion oncoprotein are not instructive for B-ALL development. If anything, we consistently observe that MLL-AF9 expression levels were lower in lymphoid clones when driven by a similar artificial promoter.

We wondered where differences in *MLL*r expression in lymphoid versus myeloid leukemias might arise from. Obviously, expression of the MLL fusion proteins is driven by the endogenous enhancers and promoters at chromosome 11 and we noted that the *MLL1* locus lies in close vicinity of the lymphoid-specific *CD3E*, *CD3D* and *CD3G* genes (Figure 3a). We therefore wondered whether this locus would be more accessible in lymphoid cells compared to myeloid cells, thereby explaining higher expression levels. Indeed, we found increased chromatin accessibility of the *MLL1* locus in lymphoid cells, along with higher accessibility around the *CD3* loci (Figure 3b, Supplemental Figure 3a). In addition, lymphoid cells had higher H3K27 acetylation (H3K27ac) depositions around the promoter regions of the *MLL1* and *CD3D* genes as detected by ChIP-seq of murine hematopoietic cells and progenitors (Figure 3c, Supplemental Figure 3b). These epigenetic landscapes aligned with higher expression levels of *MLL1* in lymphoid compartments, both at the single cell level as well as in bulk RNAseq data gene expression (Figure 3d, Supplemental Figure 3c-d).

Taken together, based on our data and those of others, we conclude that *MLL* fusion oncogenes can give rise to myeloid and lymphoid clones and that lymphoid clones can undergo a lineage switch towards a myeloid cell fate dependent on extrinsic cues. When expression is driven by an artificial promoter we consistently observe that myeloid clones express higher levels of the oncogene compared to lymphoid clones, both *in vitro* as well as *in vivo* in humanized MISTRG mouse models. When expressed under their physiological promoter and enhancer elements at the *MLL1* locus at chromosome 11 we observed increased chromatin accessibility and H3K27ac most likely explaining the higher expression levels, but this does not necessarily indicate that high MLL fusion protein expression is a requirement for transformation along the lymphoid lineage.

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Figure legends

Figure 1: Lymphoid clones maintain both lymphoid and myeloid potential in contrast to myeloid clones which are committed to their lineage

a. Pediatric *MLL-AF4* mixed phenotype leukemia primary cells were sorted for CD19$^+$ (lymphoid clone) and CD33$^+$ (myeloid clone) and cultured under myeloid-restricted and lymphoid-permissive MS5 coculture conditions (see legend Supplemental Figure 1). The lymphoid clone maintained both lymphoid and myeloid potential whereas the myeloid clone showed commitment to the myeloid lineage as determined by CD33/CD19 immunophenotype and May-Grünwald Giemsa (MGG) staining. RNA was isolated at the indicated timepoints. **b-c.** RNA sequencing was performed (using the lexogen Quantseq 3' prep kit and Illumina NextSeq500) followed by GSEA analyses comparing CD19$^+$ cells under myeloid-restricted conditions (#3) to CD19$^+$ after sorting (#2). **d-e.** Examples of B lymphoid and myeloid lineage genes **(d)**, and *MLL-AF4* target genes **(e)** from RNAseq on bulk (#1), CD19 sort (#2), CD19$^+$ cells under myeloid restricted conditions (#3) and CD33$^+$ cells under lymphoid permissive conditions (#4).

Figure 2: Lentiviral CB CD34$^+$ transduction model showed higher *MLL-AF9* expression levels in AML clones compared to B-ALL clones.

a. Representative gating strategy of 5 independent *in vitro* cord blood CD34$^+$ MLL-AF9 model experiments cultured in lymphoid permissive conditions for 10 weeks (see legends Supplemental Figures 1 and 2 for vector and culture condition information), CD33$^+$ (AML) and CD19$^+$ (B-ALL) clones are identified and the EGFP signal histogram is depicted **b.** Mean Fluorescent Intensity (MFI) of EGFP signal in AML and B-ALL. **c.** Representative gating strategy of 5 MISTRG mice showing human engraftment (CD45$^+$) of CB CD34$^+$ cells transduced with *MLL-AF9* and transforming into AML and B-ALL, both with distinct EGFP signal histograms. **d.** MFI of EGFP signal in bone marrow (BM), spleen and liver for AML and B-ALL clones. **e.** *MLL-AF9* mRNA expression measured by qPCR relative to RPL27 from *in vitro* and *in vivo* experiments sorted as depicted in Supplemental Figure 2a-b. T-test (b and e), two-way analysis of variance (ANOVA) (d), **P<0.01, ***P<0.001, ****P<0.0001

Figure 3: *MLL1* epigenetic regulation and expression show lineage dependent pattern in healthy hematopoietic cells and their progenitors. a. UCSC visualization of chromosome 11, GRCh37, showing CD3 and MLL1 loci located within

a region of 130 Kb. **b.** ATACseq data revealing increased chromatin accessibility in human lymphoid cell lineages at the *MLL1* locus (GSE96772) **c.** Myeloid cells and progenitors (CMP, GMP, monocytes and macrophages) show lower H3K27ac deposition at the *MLL1* locus compared to lymphoid (CLP, B and NK cell) and early stem cells (HSC and MPP) derived from ChIP-seq analysis of murine (C57BL/6) hematopoiesis *(*GSE59636*)* **d.** Single cell RNA sequencing of human healthy bone marrow samples (own unpublished data, Pereira-Martins et al., for mapping see Supplemental Figure 3). KMT2A expression is enriched in the lymphoid compared to myeloid lineages tested by Kruskal-Wallis with post hoc Benjamini Hochberg correction.

Figure 1

a
AML/B-ALL
MLL-AF4
RNA seq #1
CD45
FSC
CD33
CD19
CD33+ sort
CD19+ sort (RNA seq #2)
myeloid-restricted culture conditions (3weeks)
CD33
CD19
RNA seq #3
lymphoid-permissive culture conditions (3weeks)
RNA seq #4
b
top 100 B cell-specific (Novershtern)
Enrichment score (ES)
NES: -1.60
FDR q-value: 0.0
RNAseq #3 CD19+ sort and myeloid-restricted culture conditions (3 weeks)
RNAseq #2 CD19+ sort
c
top 100 Myeloid-specific (Novershtern)
NES: 1.84
FDR q-value: 0.0
d
re mRNA expression
PAX5
IGHM
CD79A
CD19
EBF1
CD72
LEF1
IGKC
CD33
ITGAM
MAFB
CD14
S100A12
S100A8
B lymphoid genes
Myeloid genes
e
FLT3
HOXA9
PBX2
PBX3
TCF4
MLL-AF4 targets
#1 Bulk
#2 CD19 sort
#3 CD19 (myeloid conditions)
#4 CD33 (lymphoid conditions)

Figure 2

a
92,0
CD45
31,8
1,87
3,00
63,3
CD33
CD19
CD33+
CD19+
GFP
b
In vitro
***
MFI GFP
10^7
10^6
10^5
10^4
CD33+
CD19+
c
16,0
CD45
89,2
GFP
8,44
0,36
7,13
84,1
CD33
CD19
CD33+
CD19+
GFP
d
In vivo
**
**
**
MFI GFP
10^7
10^6
10^5
10^4
BM
Spleen
Liver
e
****
Relative expression to CD33+ clone
(corrected for RPL27 expression)
1.00
0.75
0.50
0.25
0.00
MLL-AF9
CD33+
CD19+

Figure 3

a
GENCODE V49lift37
chr11:
118,200,000
118,250,000
118,300,000
118,350,000
118,400,000
CD3E
ENSG00000285827
TMEM25
ARCN1
CD3G
ATP5MG
IFT46
UBE4A
TTC36
CD3D
KMT2A
~ 130 K
b
Chromatin Accessibility
Distance to TSS KMT2A
-1914
-1326
-793
-94
HSC
CMP
GMP
MEP
MPP
CLP
B
CD4
CD8
ery
NK
c
Murine Mll1/Kmt2a
(H3K27ac)
Mll1
CpG: 75
CpG: 77
CpG: 119
LT-HSC
ST-HSC
MPP
MEP
CMP
GMP
Mono
Macrophage
CLP
B
NK
d
HSCs/early
Lymphoid
Myeloid
Erythroid
Dendritic
Stromal
umap_2
umap_1
p = 4.02e-7
KMT2A
Expression Level
(logReadCount)
Myeloid cells
Lymphoid cells

Supplemental Figures

MLL-AF9 expression levels do not instruct lineage fate

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Supplemental Figure 1

Supplemental Figure 1: CB MLL-AF9 B ALL model shows lineage plasticity *in vitro*. a. CB CD34$^+$ cells were transduced with UMG LV6 MLL-AF9 lentiviral vectors[1] and cells were transformed along the lymphoid lineage on MS5 bone marrow stromal cells for a period of three weeks as described in Horton et al.[2] Flow cytometry was performed for CD33 (WM53), CD19 (HIB19), CD14 (HCD14), CD15 (W6D3) and CD11b (ICRF44) markers (all from Biolegend) confirming a B-ALL phenotype. **b.** B-ALL transformed MLL-AF9 cells were plated *in vitro* under myeloid restricted conditions that block lymphoid commitment (MS5 co-culture in αMEM, 12.5% heat-inactivated (HI) fetal calf serum (FCS), 12.5% HI horse serum (HS), PenStrep, 2mM glutamine, 57.2 µM β-mercaptoethanol and 1 mM hydrocortisone (HC), supplemented with 20 ng/ml IL-3, SCF, FLT3-L, GCSF and TPO) or under lymphoid permissive conditions that allow outgrowth of both B-lymphoid and myeloid cells[2] (similar as above but without HC, HS, and IL-3 and with the addition of 10 ng/ml IL-7 and 50 µg/ml vitamin C). **c.** Cumulative cell growth for both myeloid and lymphoid conditions as described under b. **d.** LM-PCR (as previously described)[3,4] was performed on CD19$^+$ B-ALL cells at start, on cells grown under lymphoid conditions, and on cells grown under myeloid conditions as described under b-c, showing that the same clone with the identical integration site expanded under all conditions (arrow: specific PCR product, star: internal control).

Supplemental Figure 2

Supplemental Figure 2: Sorting strategy of clones for qPCR analysis. **a.** Sorting strategy on day 48 of in vitro CB MLL-AF9 model under lymphoid permissive conditions (MS5 co-culture in IMDM medium supplemented with 20% FCS, PenStrep, 1% Insulin-Transferrin-Selenium-Ethanolamine, 50µg/mL vitamin C, and cytokines IL3, IL7, SCF and FLT3-L all at 10ng/mL). **b.** CB $CD34^+$ cells were transduced with MLL-AF9 and tail vein-injected into sub-lethally irradiated (1Gy) MISTRG mice, expressing human allelles for M-CSF, GM-CSF, IL3, TPO and SIRPα.[5] At sacrifice, clones derived from liver (10L) and spleen (9L and 11R) were sorted based on gates 1 and 2 as indicated. Clones #1 and #2 represent GFP-low and GFP-high cells, respectively, distinguishing CD33 (myeloid) from CD19 (lymphoid) cells. **c-e.** Co-transduced empty vector mCherry-expressing cells (pRRL-SFFV_EV-mCherry)[6] did not show differences in MFI levels between myeloid and lymphoid clones, in vitro (c, week 10) or in vivo upon transplantation in MISTRG mice (d, histograms of BM; e, MFI quantification from BM, spleen and liver). T-test (c), two-way analysis of variance (ANOVA) (e), not significant (ns).

Supplemental Figure 3

Supplemental Figure 3: The epigenetic landscape of the CD3 locus has similarities with that of MLL1. a. ATACseq data showing increased chromatin accessibility in human lymphoid cell lineages at the *CD3D/E/G* loci (GSE96772).[7] **b.** H3K27ac deposition at the *CD3D* locus. ChIP-seq analysis of murine (C57BL/6) hematopoiesis has a similar pattern as the H3K27ac at the MLL1 locus (see Figure 3) (GSE59636).[8] **c.** Healthy bone marrow single cell RNA sequencing UMAP projection (own unpublished data, Pereira-Martins et al.) of KMT2A and lineage defining genes: Lymphoid (CD3E, CD19), Myeloid (CD14, MPO), Erythroid (GYPA) and hematopoietic stem cells (CD34), projection based on AGX Zeng et al.[9] **d.** MLL1 mRNA levels in human erythroid and myeloid cells/progenitors are lower than in lymphoid counterparts (GSE24759).[10]

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