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MLKL promotes cellular differentiation in myeloid leukemia by facilitating endosomal secretion of G-CSF

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous condition characterized by the blockade of cellular differentiation and the suppression of cell death. The failure of a coordinated cellular differentiation in AML is largely attributed to the disruption of normal lineage-specific transcription factor function can controls cellular differentiation. However, alternative mechanisms of cellular differentiation programs in the hematopoietic systems and specifically in AML remain largely unexplored. Here we report that mixed lineage kinase domain like protein (MLKL) contributes to the cellular differentiation of hematopoietic progenitor cells at steady-state, in AML and during inflammation. Mechanistically, MLKL promotes the secretion of granulocyte colony stimulating factor (G-CSF) via the endosomal secretory pathway. *Mlkl*$^{-/-}$ hematopoietic stem/progenitor cells secrete reduced amounts of G-CSF while retaining their capacity of G-CSF mRNA expression, protein translation and G-CSF receptor signaling. AML patients present with reduced MLKL expression specifically in poor risk patients, which is further pronounced in immature AML subtypes (FAB M0-M2). In summary, our data show a critical contribution of MLKL to cytokine secretion and subsequent myeloid differentiation in myeloid leukemia and during inflammation.

CONTENTS

1 BACKGROUND AND INTRODUCTION

1.1 ACUTE MYELOCYTIC LEUKEMIA

In adults the acute myelocytic leukemia is the most common type of leukemia and the most common cancer in children younger than 15 years old (Short, Rytting, & Cortes, 2018), based on national cancer institute, the number of new cases of acute myeloid leukemia was 4.3 per 100,000 men and women per year and the 5 year survival rate is only 28.3% (Lin et al., 2019). Acute myeloid leukemia (AML) is characterized by the accumulation of genetic aberrations in hematopoietic progenitor cells (Short et al., 2018; Tenen, 2003). The repression of myeloid differentiation and cell death represent two critical hallmarks of AML (Hanahan & Weinberg, 2011; Jost & Hockendorf, 2019).

1.2 RISK CLASSIFICATION

Thanks to the development of molecular biology and genomics, the distinguish of mutated oncogenes or chromosome variation also plays an important role in AML classification nowadays, for example, FLT3-ITD mutation with high allelic burden associated with a worse prognosis. Based on these mutations or chromosome abnormality AML patients were classified into different risk groups (Favorable, Intermediate, Adverse). In the past only cytogenetic abnormality was used for risk classification (Mrozek, Heinonen, de la Chapelle, & Bloomfield, 1997; Raimondi et al., 1999), and clinical doctor soon found the patients with same cytogenetics background could have very different clinical phenotype, response to the treatment or prognosis, then recent years with the development of sequence more and more oncogenes were founded, genomics was introduced to the AML risk classification (Patel et al., 2012).

Table 1, Genomic risk stratification of acute myeloid leukemia

	Favorable	Intermediate	Adverse
Cytogenetic	t(8;21)(q22;q22.1) for RUNX1-RUNX1T1, and inv(16)(p14.1q22) or t(16;16) (p13.1;q22) for CBFB-MYH11	t(9;11)(p21.3;q23.3) for MLL3-KMT2A, and cytogenetic abnormalities not classified as favorable or adverse	t(6;9)(p23;q34.1) for DEK-NUP214; t(v;11q23.3) for KMT2A rearranged; t(9;22)(p34.1;q11.2) for BCR-ABL1; inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) for GATA2 and MECOM (EVI1); –5 or del(5q), –7, and-17/abn(17p); complex karyotype; and monosomal karyotype
Molecular	Mutated NPM1 without FLT3-ITD or with FLT3-ITD^{low}, and biallelic mutated CEBPA	Mutated NPM1 and FLT3-ITD^{high}, and wild-type NPM1 without FLT3-ITD or with FLT3-ITD^{low} (without adverse-risk genetic lesions)	Wild-type NPM1 and FLT3-ITD^{high}, mutated RUNX1, mutated ASXL1, and mutated TP53

The table is adapted from Nicholas J Short and colleagues, Acute myeloid leukemia, Lancet. 2018 Aug 18;392(10147):593-606 (Short et al., 2018). ITD=internal tandem duplication.

1.3 DIAGNOSIS AND TREATMENT OUTLINES OF ACUTE MYELOCYTIC LEUKEMIA

Leukemia is a disease of the bone marrow hematopoietic stem cells and progenitor cells, so a bone marrow biopsy and aspiration are needed to confirm the diagnosis, a diagnosis of acute myeloid leukemia requires identification of 20% or more myeloid blasts with a morphological assessment of the peripheral blood or bone marrow (Estey & Dohner, 2006; Short et al., 2018). For further confirming the type of leukemia, additional special examinations are needed to accurately classify subtypes of leukemia and give the most appropriate treatment

plan which includes: special staining, flow cytometry, and chromosome inspection (Dohner et al., 2017).

Because of the complexity of leukemia classification and prognosis, there is no “one-size-fits-all” treatment strategy that requires clinical doctors customize a treatment plan for every patient based on AML subtype, risk classification and some mutation or genetic abnormal which can be targeted (Arber et al., 2016). At present, the main treatment methods are chemotherapy, radiation therapy, targeted therapy, immunotherapy, hematopoietic stem/progenitor cell (HSPC) transplantation. Due to the development of target therapy and immunotherapy treatment, the prognosis of leukemia has been greatly improved, so a considerable number of patients can be cured or long-term stable, the era of leukemia is an "incurable disease" has gone.

1.4 THE SPECTRUM OF GENETIC ABERRATION IN ACUTE MYELOCYTIC LEUKEMIA

The number of mutations per acute myeloid leukemia genome or exome is lower than most other cancer types with an average of only five recurrent mutations per acute myeloid leukemia genome (Lawrence et al., 2014), but at least one driver mutation can be found in 96% de-novo acute myeloid leukemia, and 86% of patients have two or more driver mutations (Papaemmanuil et al., 2016; Short et al., 2018). With different permutation and combination of mutations in each acute myeloid leukemia patients, make the prognosis and treatment strategy are very heterogeneity between different acute myeloid leukemia patients. Some mutations serve as prognosis markers and also targets for acute myeloid leukemia targeting therapies (Pratcorona et al., 2013).

1.5 CLINICALLY-RELEVANT AML-SPECIFIC ONCOGENIC DRIVER GENES

1.5.1 FLT3-ITD

Mutations of the FMS-like tyrosine kinase 3 (FLT3, also known as CD135) gene occur in approximately 30% of all AML cases, and the internal tandem duplication (ITD) representing

the most common type of FLT3 mutation (FLT3-ITD) (Daver, Schlenk, Russell, & Levis, 2019). FLT3 is a transmembrane ligand-activated receptor tyrosine kinase, expressed on the membrane of $CD34^{+}$ primitive hematopoietic cells and has a crucial role in many regulatory processes of early hematopoietic cells (Kikushige et al., 2008; Kindler, Lipka, & Fischer, 2010; Maroc et al., 1993; Rosnet et al., 1993). FLT3-ITD^{High} is associated with a high leukemic burden and worse prognosis in AML patients (Ding et al., 2012; Grimwade & Mrozek, 2011; Khaled, Al Malki, & Marcucci, 2016; Thiede et al., 2002; Whitman et al., 2001), but he first clinical use of FLT3 inhibitors, quickly resulted in the resistance-conferring FLT3 point mutations, which highlighting FLT3-ITD mutations as driver oncogene (Smith et al., 2012). But scientists keep working on FLT3 inhibitors, the next-generation FLT3 inhibitors (for example Gilteritinib) have already shown promising clinical outcomes (Perl et al., 2017).

1.5.2 AML-ETO

AML-ETO also is known as RUNX1-RUNX1T1 fusion oncoprotein, in the t(8;21) (q21;q22) translocation RUNX1 - Runt related Transcription Factor 1 is fused with ETO (MTG8 also known as RUNX1T1) (Reikvam, Hatfield, Kittang, Hovland, & Bruserud, 2011). RUNX1 is a transcription factor that regulates the differentiation of hematopoietic stem cells into mature blood cells (Okuda, Nishimura, Nakao, & Fujita, 2001). AML-ETO is one of the most common genetic abnormal, accounts for 15% of all acute myeloid leukemia (AML) cases and up to 40% of those classified by the French-American-British system as M2 subtype (K. Petrie & Zelent, 2007), and in AML, RUNX1-RUNX1T1 demonstrate a high number of secondary genetic lesions, KIT, NRAS, and ASXL1 are the most common mutations combined with RUNX1-RUNX1T1 (Eder et al., 2013).

1.5.3 MLL-ENL

MLL also is known as KMT2A (lysine methyltransferase 2A) is a transcriptional coactivator that plays an essential role in regulating gene expression during early development and hematopoiesis(Eguchi, Eguchi-Ishimae, & Greaves, 2003). The chromosomal translocation of t(11;19) (q23;p13.3) induced the fusion oncoprotein of MLL-ENL (Fu, Liang, & Shih, 2007), several studies revealed due to refractoriness to chemotherapy and a shorter period to

relapse, MLL-ENL positive is related with poor prognosis (Dimartino & Cleary, 1999; Kotani et al., 2019; Meyer et al., 2013).

1.6 PROGRAMMED CELL DEATH

Programmed forms of cell death repress leukemogenesis by killing transformed cells. In addition, inflammatory forms of programmed cell death also exert their tumor-suppressive function by eliciting an inflammatory signaling program (Jost & Hockendorf, 2019). The inflammation results in the propagation of a myeloid differentiation program based, at least in part, on the release of IL-1ß (Interleukin-1ß) from AML blast cells in response to oncogenic signaling effectively repressing leukemogenesis (Hockendorf et al., 2016). Both mechanisms, (i) induction of cell death and (ii) the inflammation-driven myeloid differentiation of the AML stem and blast cells provide a powerful molecular mechanism of disease repression (Jost & Hockendorf, 2019) (Hockendorf et al., 2016).

Independent of the RIPK3-dependent signaling, conventional BCL-2 (B-cell lymphoma 2) regulated apoptotic cell death of leukemia initiating cells (LICs) and blast cells also controls the leukemia biology. The clinical response of patients to the induction chemotherapy and even the relapse rate after allogeneic stem cell transplantation can be predicted by the level of mitochondrial priming present in AML cells at initial diagnosis (Vo et al., 2012). An elevated level of mitochondrial priming, representing cellular stress mediated by the BCL-2 family, defines patients with a better response rate to induction chemotherapy compared to patients with low levels of mitochondrial priming (Vo et al., 2012). The central contribution of the BCL-2 family to the hematopoietic progenitor cell integrity is also exemplified by the critical role of MCL-1 (Myeloid Cell Leukemia 1) in both healthy and malignant progenitor cells (Anstee et al., 2019; Glaser et al., 2012; Vo et al., 2012). These examples provide evidence that the coordinated induction of both non-apoptotic as well as apoptotic cell death represent a critical roadblock to malignant transformation and therapy resistance in AML patients.

Apoptotic cell death is controlled by the interaction of proteins of the BCL-2 family, which can be divided into a pro-apoptotic and a pro-survival subgroup (Adams & Cory, 2007; Chipuk, Moldoveanu, Llambi, Parsons, & Green, 2010; Strasser, Cory, & Adams, 2011). When

apoptosis is induced, pro-apoptotic BAX (BCL2 Associated X)/BAK (BCL2 Antagonist)-like proteins act as effector proteins by permeabilizing the outer mitochondrial membrane. Consequently apoptotic factors such as cytochrome c are released into the cytoplasm leading to the activation of caspases executing inflammatory-silent apoptosis by selectively cleaving cellular target proteins (Pop & Salvesen, 2009).

In contrast, necroptotic cell death proceeds independently of effector caspases and provides a substantial inflammatory stimulus, which is mediated by damage-associated molecular patterns (DAMPs) released from dying cells (Galluzzi & Kroemer, 2008; Peltzer & Walczak, 2019) eventually resulting in the activation of an immune response (Yatim et al., 2015). The central mediator of necroptotic cell death is RIPK3 and its downstream effector molecule MLKL. Upon upstream activation, RIPK3 phosphorylates MLKL within the pseudo-kinase domain resulting in the unleashing of the N-terminal four-helix bundle domain (4HB) of MLKL. This activated form of MLKL then translocates to the membrane, oligomerizes and forms pores thereby mediating the release of cytoplasmic content into the extracellular space (Murphy et al., 2013; Sun et al., 2012; Tanzer et al., 2015; Vucur et al., 2018).

1.7 CHARACTERISTICS OF MLKL

Cell death is essential for the development of multicellular organisms, maintenance of homeostasis, and defense against microbial infection (Dovey et al., 2018; Fiers, Beyaert, Declercq, & Vandenabeele, 1999). Mixed lineage kinase domain-like (MLKL) is a component of the "necrosome," the complex that triggers tumor necrosis factor (TNF)-induced cell death by necroptosis, and MLKL phosphorylation by RIPK3 constitutes a critical checkpoint in necroptosis signaling (Murphy et al., 2013). MLKL locate on chromosome 16, q23.1, contain 12 exons, MLKL comprises an N-terminal four-helix bundle (4HB) domain connected to a C-terminal PsKD via a two-helix linker, which termed the brace helices (Murphy et al., 2013; E. J. Petrie et al., 2018). Because the kinase like domain of MLKL lacks two of the three conserved catalytic residues which are important for phosphoryl transfer activity, thus MLKL is classified as a ‘‘pseudokinase’’ (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). Upon initiated by receptor-interacting serine/threonine-protein kinase 3 (RIPK3) dependent

phosphorylation, MLKL oligomerization result in necroptosis forming pores that leak intracellular contents, such as cytokines, chemokines, and other intracellular proteins (Sun et al., 2012; Vucur et al., 2018).

2 MATERIALS AND METHODS

2.1 *MLKL*$^{-/-}$ AND WILDTYPE (WT) C57BL/6 MICE

The *Mlkl*$^{-/-}$ and wildtype (WT) C57BL/6 mice were used, the *Mlkl*$^{-/-}$ mice have been described previous (Murphy et al., 2013). Mice at 8-9 weeks of age were used for bone marrow cell collection from at least three mice of the same age for each experiment (Hockendorf et al., 2016). Animals were maintained under specific pathogen-free conditions, and all animal experiments were approved by the District Government of Upper Bavaria.

2.2 PRIMARY BONE MARROW CELLS

The primary bone marrow cells were harvested from mice (femurs and tibias) of indicated genotypes 4 days after injection of 150 mg/kg 5-fluorouracil (5-FU; Sigma) intraperitoneally and cultured in RPMI medium containing 20% FCS (Fetal Calf Serum) supplemented with growth factors (10 ng/ml IL-3, 10 ng/ml IL-6; R&D Systems, 100 ng/ml SCF; eBioscience).

2.3 COLONY FORMATION ASSAY

For colony formation assay, all duplicate cultures were performed in 35mm petri dishes with MethoCult^{TM} GF M3434 medium (STEMCELL Technologies Inc.), GFP^{+}/Lin^{-} (green fluorescent protein positive/ lineage negative) bone marrow cells were sorted and seeded at density of 2500 cells/plate, after 10 days the colonies were counted by light microscopy.

2.4 PLASMIDS AND RETROVIRUS PREPARATION

The FLT3-ITD (FMS-like tyrosine kinase 3-internal tandem duplication), MLL-ENL (mixed lineage leukemia-eleven nineteen leukemia translocation), and AML-ETO (RUNX1/RUNX1T1

translocation) retroviral plasmids have been previously described in our lab; retrovirus supernatants were produced in the packaging cell line Phoenix by using Metafectene™ Pro transfection, pMIG empty plasmid served as a control (Hockendorf et al., 2016).

2.5 FLOW CYTOMETRY (FACS)

As standard FACS staining protocol described in previous paper (Hockendorf et al., 2016), cells were washed and resuspended at a concentration of 1×10^6 cells/ml. Then cells were pre-incubated with Fc-block and subsequently stained with fluorescently labeled antibodies as listed in the antibody list. PI (Propidium Iodide; Invitrogen) staining was used for viability gating. Flow analysis was performed on a BD FACS Canto II (BD Biosciences) and for GFP sorting BD FACSAria™ III cell sorter was used; data were analyzed using FlowJo™. For intracellular staining, the cells were fixed with 4% paraformaldehyde (pH=7) in PBS (room temperature, 10 min), then permeabilized with 0.1% Triton-X100 for 5 min in room temperature and blocked with 3% FBS. After that cells were incubated 1 hours with the indicated antibodies and then with secondary antibodies. For supernatant cytokine measurement cytometric bead array (CBA; BD Biosc.) were used according to manufacturer's instructions and performed in triplicate.

Table 2, antibody list

	Antibody	**Clone/Lot.**	**Supplier**
Lineage marker	IL-7Ra	A7R34	eBioscience
	B220	RA3_6B2	eBioscience
	CD19	eBio1D3	eBioscience
	TCR-b	H57-597	eBioscience
	CD3	17A2	eBioscience
	CD11b	M1/70	eBioscience
	Gr-1	RB6-8C5	eBioscience
	Ly6C	HK1.4	eBioscience

	TER-119	TER-119	eBioscience
Progenitor	Sca-1	D7	eBioscience
	c-Kit	2B8	eBioscience
	CD34	700011	R & D systems
	CD11/32	93	eBioscience
Others	EEA1	C45B10	CST
	G-CSF	MAB414	R & D systems
	Rabbit Anti-Mouse IgG	A-21427	Invitrogen
	Donkey Anti-Rabbit IgG	ab175649	Abcam

2.6 FLUORESCENCE CONFOCAL MICROSCOPY

The WT or *MLKL*$^{-/-}$ bone marrow cells were seeded in the chamber slide in density of 1×10^6/ml for 2 days (with cytokine cocktail, 10 ng/ml IL-3, 10 ng/ml IL-6; R&D Systems, 100 ng/ml SCF; eBioscience), for immunofluorescence staining, the cells were fixed with 4% paraformaldehyde (pH=7) in PBS in room temperature for 10 min, then permeabilized with 0.1% Triton-X100 for 5 min in room temperature and blocked with 3% FBS. After that cells were incubated 1 hours with the indicated antibodies and then with secondary antibodies (in antibody list). Images of the immune-stained cells were captured with a white light laser confocal microscope (TCS SP8 X, Leica Microsystems). The co-localization between EEA1 and G-CSF was calculated with Leica Application Suite X software (Ver.3.4.2, Leica).

2.7 RNA ISOLATION AND REAL-TIME PCR

The total RNA isolation was performed by Nucleospin®RNA (MACHEREY-NAGEL), following manufacturer instruction. RNA concentration and purity were determined using the NanoDrop spectrophotometer (NanoDrop Technologies). 1μg total RNA was reverse transcribed to 20μl cDNA by SuperScript II Reverse Transcriptase (Life Technologies), The qPCR was performed using LightCycler® 480 (Roche) Real-Time PCR System, the reaction protocol was 95 °C for 10 min, followed by 40 cycles of 92°C for 15 sec and 62.5°C for 1 min. Every

sample was in triplicate and the expression was normalized to the reference gene expression using the $2^{\Delta\Delta Ct}$ method, all the primers in list below.

Table 3, Primer list

	sense	**antisense**
mG-CSF	GCTGCTGCTGTGGCAAAGT	AGCCTGACAGTGACCAGG
mGAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

2.8 STATISTICAL ANALYSIS AND DATA SOURCE

Data are presented as the mean ± SD or SEM and analyzed using a student's t-test (two-tailed) or one-way ANOVA with post-hoc analysis. And *P<0.05, **P<0.005, ***P<0.0005 were calculated by SPSS Statistics (version 20.0, IBM Corp) or GraphPad Prism (version 7.05, GraphPad Software, Inc). The level 3 TCGA Acute Myeloid Leukemia (LAML) datasets containing 173 patients' samples were downloaded for clinical analysis, the "count" is the RSME normalized mRNA count(Li & Dewey, 2011), which represents TCGA mRNA expression the unit for gene expression is "$\log_2$(count+1)". For GSEA and heatmap, the latest official tool was downloaded from http://software.broadinstitute.org/gsea (Ver. 3.0) (Subramanian et al., 2005).

3 THE AIM OF THE STUDY

Acute myeloid leukemia (AML) is characterized by accumulation of primitive myeloid cells including abnormally differentiated progenitors (blasts) with impairment of normal hematopoiesis, which lead to severe complications (Short et al., 2018; Tenen, 2003). Restricting cell death and differentiation is the hallmarks of AML. The classic programed cell death, apoptosis is characterized as an almost-invisible corpse that is soon consumed by neighbor cells (Hanahan & Weinberg, 2011). Like apoptosis the programed cell necrosis, termed necroptosis is under genetic control. However, necroptosis doesn't display a stereotyped morphology and it normally release damage-associated molecular patterns

(DAMPs), which induce inflammation (Galluzzi & Kroemer, 2008; Peltzer & Walczak, 2019). It has been reported RIKP3 restricts the myeloid leukemogenesis (Hockendorf et al., 2016). RIPK3 is a key mediator in tumor necrosis factor receptor (TNFR) signaling pathway (Pasparakis & Vandenabeele, 2015), upon activation, RIPK3 directly target MLKL which is the executor of necroptosis (Cai et al., 2014). Recently, Yoon et al. reported MLKL contributed to the endosomal trafficking independent of the necroptosis function (Yoon, Kovalenko, Bogdanov, & Wallach, 2017). To elucidate the new mechanism independent of necroptosis, we sought to examine the role of MLKL in AML and how MLKL affects differentiation within the myeloid lineage. Here, we report that MLKL mediates the release of bio-active granulocyte colony stimulating factor (G-CSF) via the endosomal pathway thereby effectively controlling myeloid progenitor differentiation during steady-state, during conditions of inflammation as well as during myeloid leukemia.

4 RESULTS

4.1 AML PATIENTS SHOW SIGNIFICANTLY REDUCED MLKL EXPRESSION

Based on the critical tumor suppressive function of RIPK3 during leukemogenesis (Hockendorf et al., 2016), we determined the role of its downstream effector MLKL in AML. We first characterized gene expression levels of MLKL in human AML samples by comparing gene expression profiles of 542 human AML bone marrow (BM) samples with 73 healthy BM controls (GSE13204) (Haferlach et al., 2010; A. Kohlmann et al., 2008; Kuhnl et al., 2010). We found that MLKL expression was significantly reduced in AML BM as compared to healthy BM controls (Figure 1A).

This finding was corroborated in an independent dataset (GSE15061) that compared 202 AML patients to 164 samples from patients diagnosed with myelodysplastic syndromes (MDS) to 69 healthy control BM samples. Whereas MLKL expression remained unchanged between MDS (various risk categories) and healthy controls, the expression was significantly repressed in the AML patient cohort (Figure 1B).

We next explored the MLKL expression based on the clinical risk category of AML patients. We subdivided patients from the TCGA AML dataset (TCGA-LAML, The Cancer Genome Atlas, Leukemia_Acute Myeloid Leukemia, 20171013) according to their clinical risk classification and we found that patients with favorable risk (n=32) have significantly higher MLKL expression compared to patients in the intermediate (n=103) or poor (n=36) risk categories (One-way ANOVA with post-hoc analysis) (Figure 1C). Even more intriguing was the observation that AML patients grouped according to the French-American-British (FAB) classification system substantially differed in MLKL expression. We found the most immature samples (FAB M0-M2) to have significantly reduced MLKL expression compared to more differentiated AML subgroups (FAB M3-M5) as well as compared to normal bone marrow (BM) samples (Figure 1E).

Of note, differences in MLKL was not differentially expressed when grouping the patients according to their genomic alterations (Figure 1F). Only CEBP α -mutated patient samples presented with a significantly reduced expression level of MLKL (Figure 1F).

These data suggested that MLKL might serve as a tumor-suppressor in AML as expression levels were significantly reduced in AML patients compared to healthy BM and in intermediate and high-risk patients compared to low-risk AML patients. Yet, screening of the TCGA dataset for AML (n=197) failed to show any coding mutations in the *MLKL* gene in AML patients and only one mutation was found in the TCGA dataset for AML in the PanCancer cohort (Figure 1.1A). Across all cancer types, no mutational hotspot was observed in MLKL (Figure 1.1B) (Cerami et al., 2012; Gao et al., 2013).

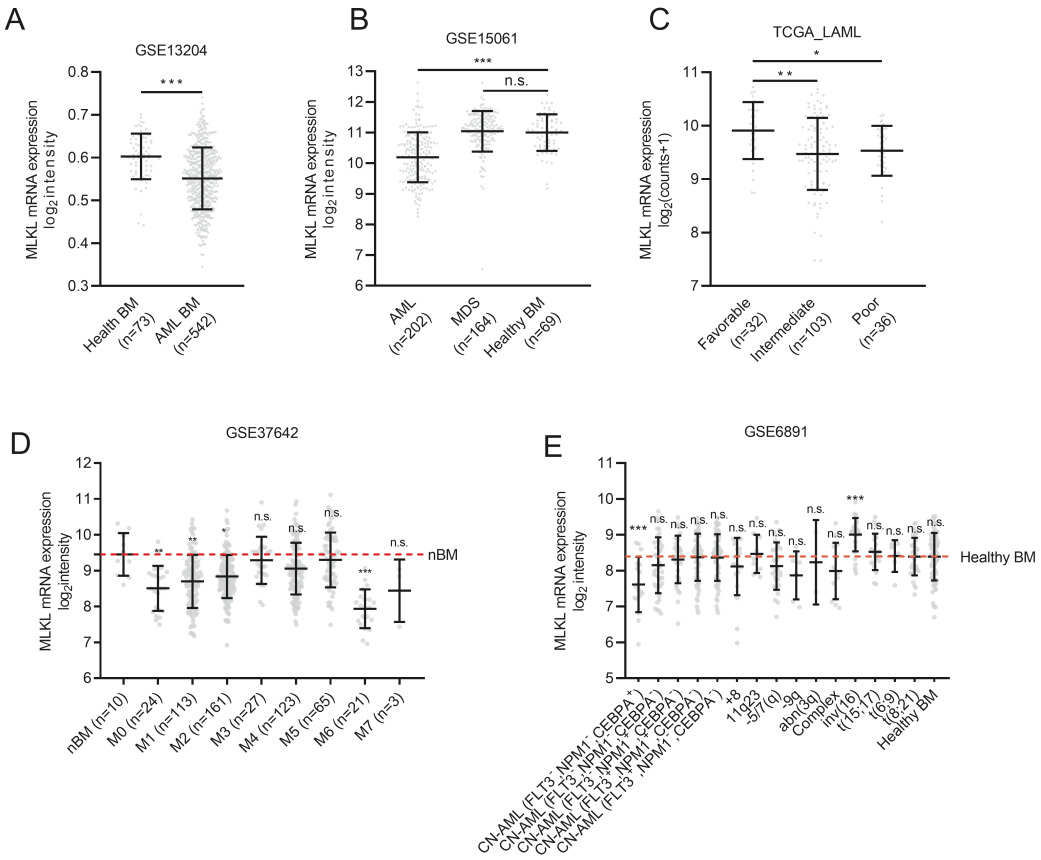

Figure 1, AML patients show significantly reduced MLKL expression

A, data from the GSE13204 (GPL570 platform) indicate MLKL expression is lower in AML samples compared to healthy controls (t-test, mean with SD).

B, data from the GSE15061 indicate MLKL expression in MDS and healthy controls compared to AML patients, and MLKL expression is lower in AML compared to healthy control, but no significant MLKL expression difference was found between MDS and healthy control (one-way ANOVA with post-hoc tests, mean with SD).

C, data from the TCGA AML cohort (TCGA_LAML) categorized by clinical risk category, compared with intermediate and poor risk patients, the patients with favorable risk have higher MLKL expression (one-way ANOVA with post-hoc tests, mean with SD).

D, data from GSE37642 display the MLKL expression in different AML subtypes based on FAB system, We found the most immature samples (FAB M0-M2) to have significantly reduced MLKL expression compared to more differentiated AML subgroups (FAB M3-M5).

E, data from GSE6891 display the MLKL expression in different AML subtypes based on different genomic abnormal, only CEBP-α-mutated patient samples presented with a significantly reduced expression level of MLKL.

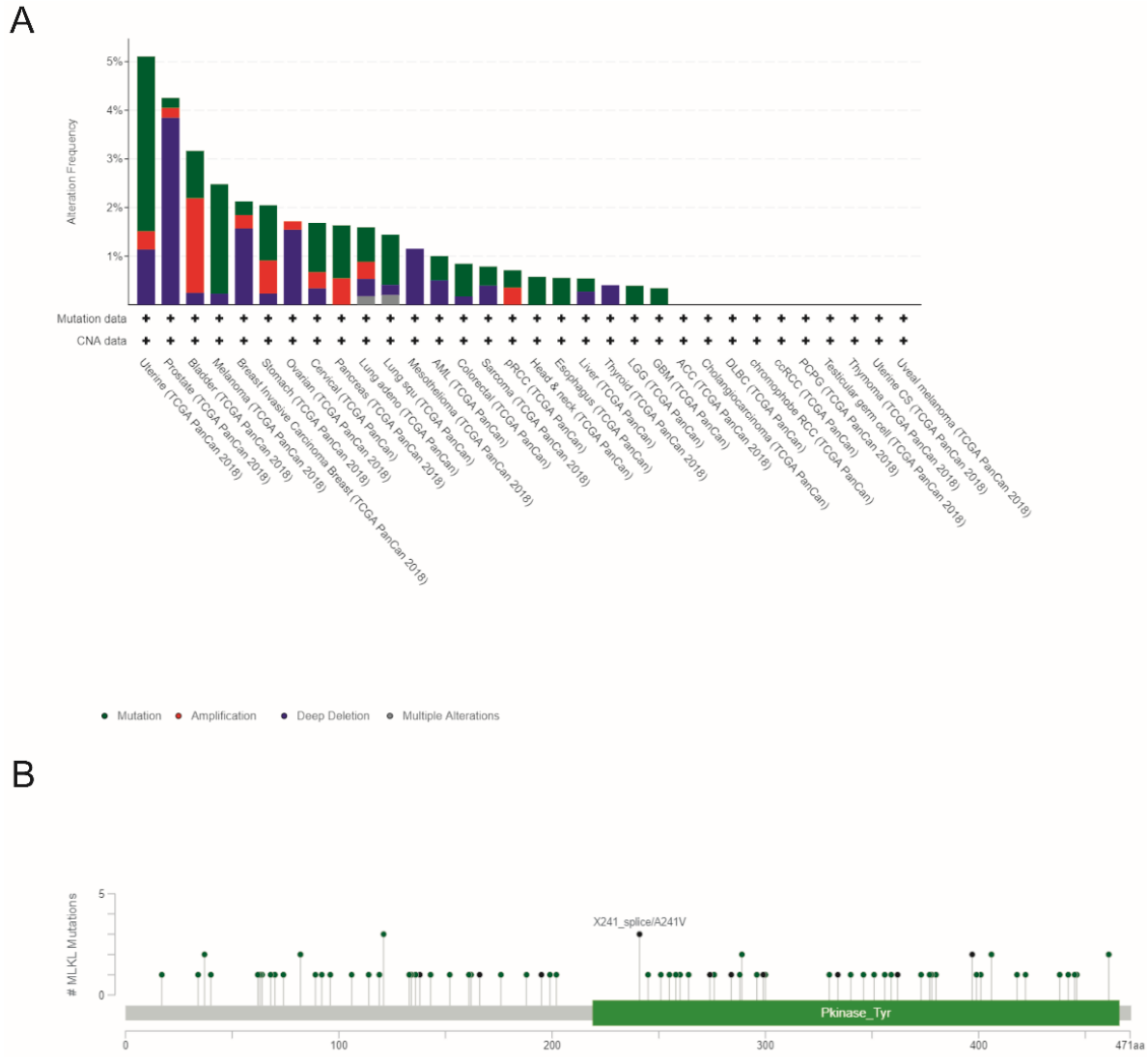

Figure 1.1. Low mutational rate of MLKL in AML

To evaluate the mutation frequency and location of variants in MLKL in different cancers the cBioPortal for Cancer Genomics (http://cbioportal.org) was queried for MLKL in 09/2019.

A, bar graphs display the frequency of genetic alterations of MLKL in various cancers analyzed by using TCGA data.

B, lolli plot visualizing the distribution of MLKL alterations identified in various cancers analyzed by the TCGA within the protein sequence of MLKL, and no mutation hot spot was identified.

4.2 MLKL PROMOTES DIFFERENTIATION OF MYELOID LEUKEMIA STEM/PROGENITOR CELLS

Based on these findings, we postulated that loss of MLKL might promote leukemogenesis. To investigate this, we expressed several clinically-relevant patient-derived common AML driver oncogenes in primary murine hematopoietic stem or progenitor cells (HSPC) *ex-vivo*. Upon retroviral transduction, we generated *Mlkl*$^{-/-}$ or WT primary leukemic cells using AML-ETO, FLT3-ITD or MLL-ENL as driving oncogenes. After five days of culture, we characterized the number of primitive myeloid cells defined as either lineage-negative (Lin$^-$) myeloid cells or myeloid progenitor cells (Lin$^-$ Sca1$^-$ c-Kit$^+$) (containing common myeloid progenitors [CMP]; granulocyte-macrophage progenitors [GMP]; and megakaryocyte-erythroid progenitors [MEP]).

Our data revealed that loss of MLKL resulted in an expansion of all measured progenitor populations in primary murine bone marrow (Figure 2A-C). This expansion was mainly driven by increased numbers of common myeloid progenitor (CMP) implying that MLKL contributed to the myeloid differentiation of progenitor cells in AML (Figure 2D-F). Of note, contrary to the conventional concept of MLKL as pro-death protein in the necroptotic signaling cascade (Murphy et al., 2013), we observed no difference in the viability of *Mlkl*$^{-/-}$ or WT cells (Figure 2.1A-C).

To explore the possibility that the increased number of primitive *Mlkl*$^{-/-}$ progenitors might be explained by the inability of *Mlkl*$^{-/-}$ to differentiate into a specific myeloid lineage, we tested their colony-forming capacity. In line with the data obtained by flow cytometry, *Mlkl*$^{-/-}$ colonies showed an accumulation of the oligopotential myeloid progenitor population as measured by an increase in the CFU-GEMM (colonies containing granulocyte, erythrocyte, monocyte, megakaryocyte progenitors) colonies compared to WT colonies (Figure 2G-I).

In summary, the loss of MLKL resulted in a marked expansion of primitive myeloid progenitor cells and primitive hematopoietic colonies without affecting cellular survival of AML cells suggestive of a role of MLKL in myeloid progenitor differentiation in leukemia.

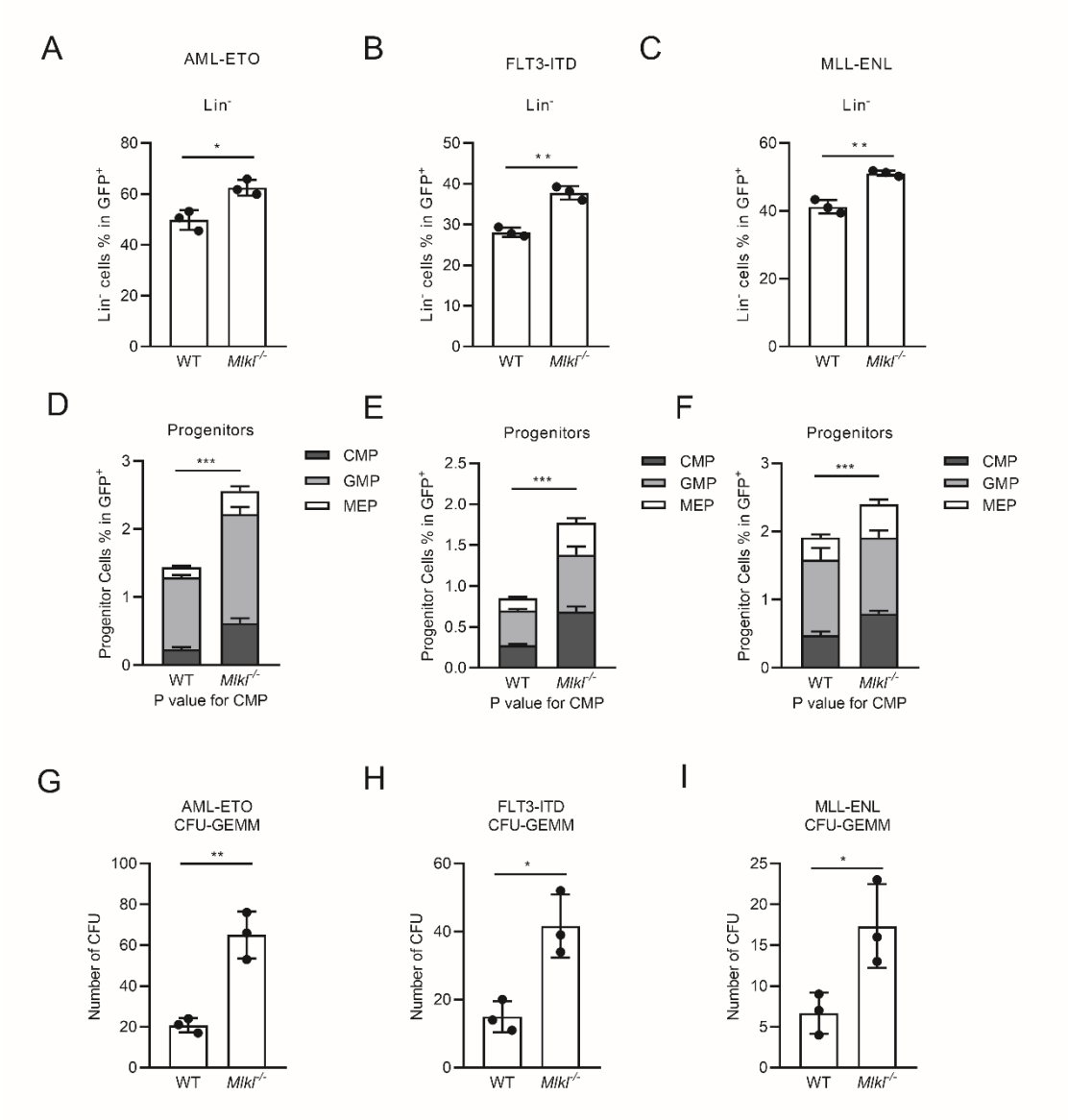

Figure 2. MLKL deletion restricts malignant myeloid differentiation

A-C, shown is the percentage of lineage negative cells (Lin-) in GFP positive cells (GFP+) of 5-FU challenged bone marrow cells (WT and Mlkl-/-) transduced with AML-ETO, FLT3-ITD or MLL-ENL (t-test, mean with SD). Our data indicate compared with WT bone marrow cells the *Mlkl*$^{-/-}$ have significantly expanded Lin- populations in all three different oncogenes.

D-F, shown is the percentage of progenitor cells in GFP+ cells, our data indicate compared with WT bone marrow cells the *Mlkl*$^{-/-}$ have significantly expanded progenitor cells population in all three different oncogenes, and CMP the most primitive type of progenitors contributes the most to the expansion. (t-test, mean with SD).

G-I, shown is the colony count of the oligopotential myeloid progenitor population CFU-GEMM flow-sorted from *Mlkl*$^{-/-}$ and WT GFP+/Lin- bone marrow cells after 10 days of culture in methocult media, *Mlkl*$^{-/-}$ groups have significantly higher colony numbers compare to WT controls (t-test, mean with SD).

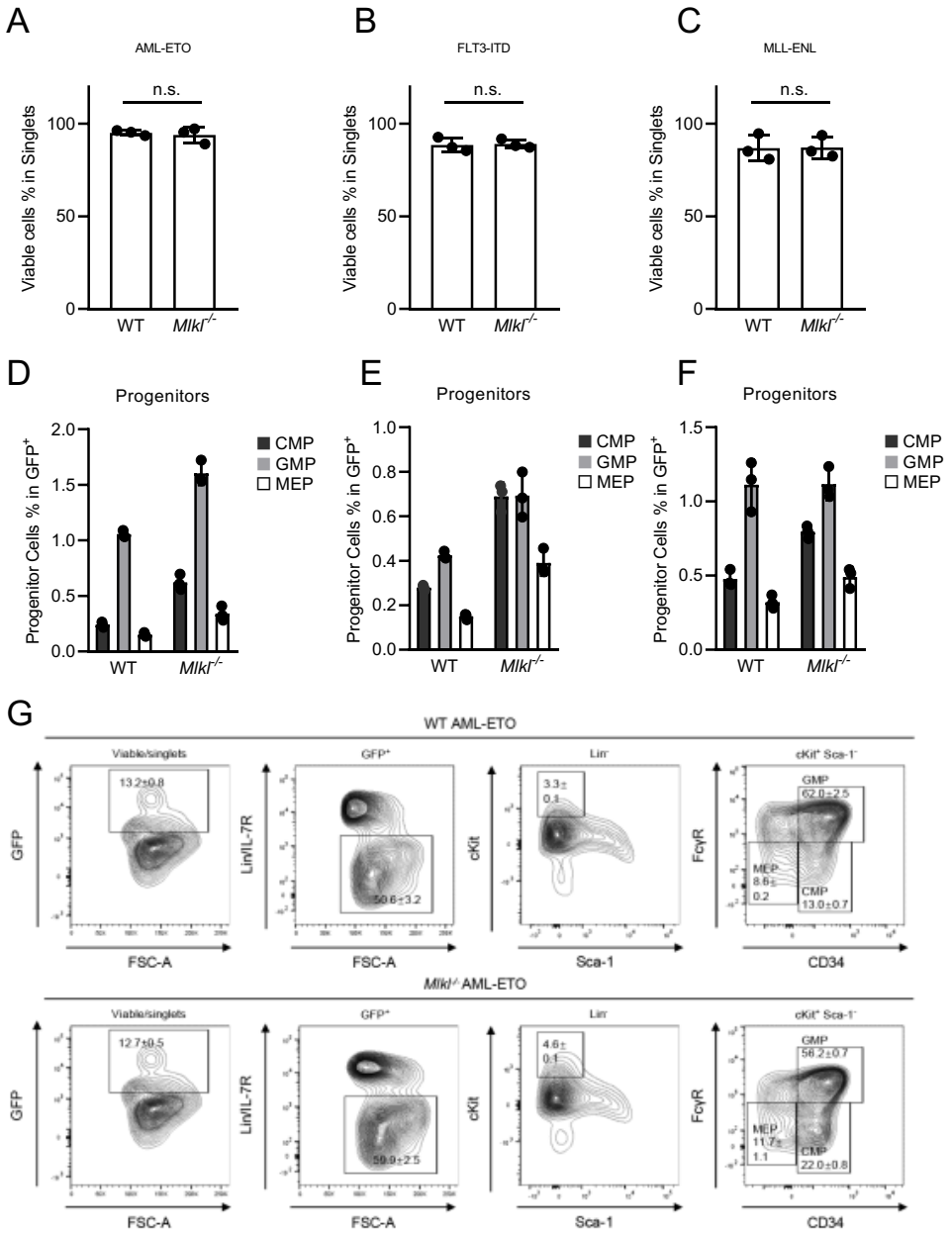

Figure 2.1. MLKL fails to contribute to cell death in AML bone marrow cells

A-C, the viability data from PI staining in singlet cells indicate no significate viability difference was found between *Mlkl*$^{-/-}$ and WT group in all three oncogene groups linked to Figure 2A-F (t-test, mean with SD). D-F, plots for the progenitor populations linked to Figure 2D-F, indicating CMP (common myeloid progenitors) population contributes the most to the expansion of progenitors populations. G, the example FACS plots shown the progenitor cell staining.

4.3 MLKL EXPRESSION CORRELATES WITH AN INFLAMMATORY RESPONSE SIGNATURE IN HUMAN AML

As MLKL promotes differentiation of myeloid progenitor cells along the granulocytic lineage in different models of AML, we next explored the downstream mechanism of this effect in the hematopoietic system. Using the TCGA dataset for AML (TCGA_LAML, 20171013) including

173 AML patient samples, we selected the samples with the top 10% highest and bottom 10% lowest MLKL mRNA expression (17 samples in each group) (Wang et al., 2019). We then submitted the transcriptome data of these samples for Gene Set Enrichment Analysis (GSEA) to distinguish differentially expressed gene signatures. The GSEA results showed a significant correlation between MLKL expression and several inflammatory response pathways (Figure 3A). Specifically, we identified a significant correlation for TNF signaling, complement signaling, interferon-γ signaling and the inflammatory response signature (Figure 3B, Table 1 and Figure 3.1).

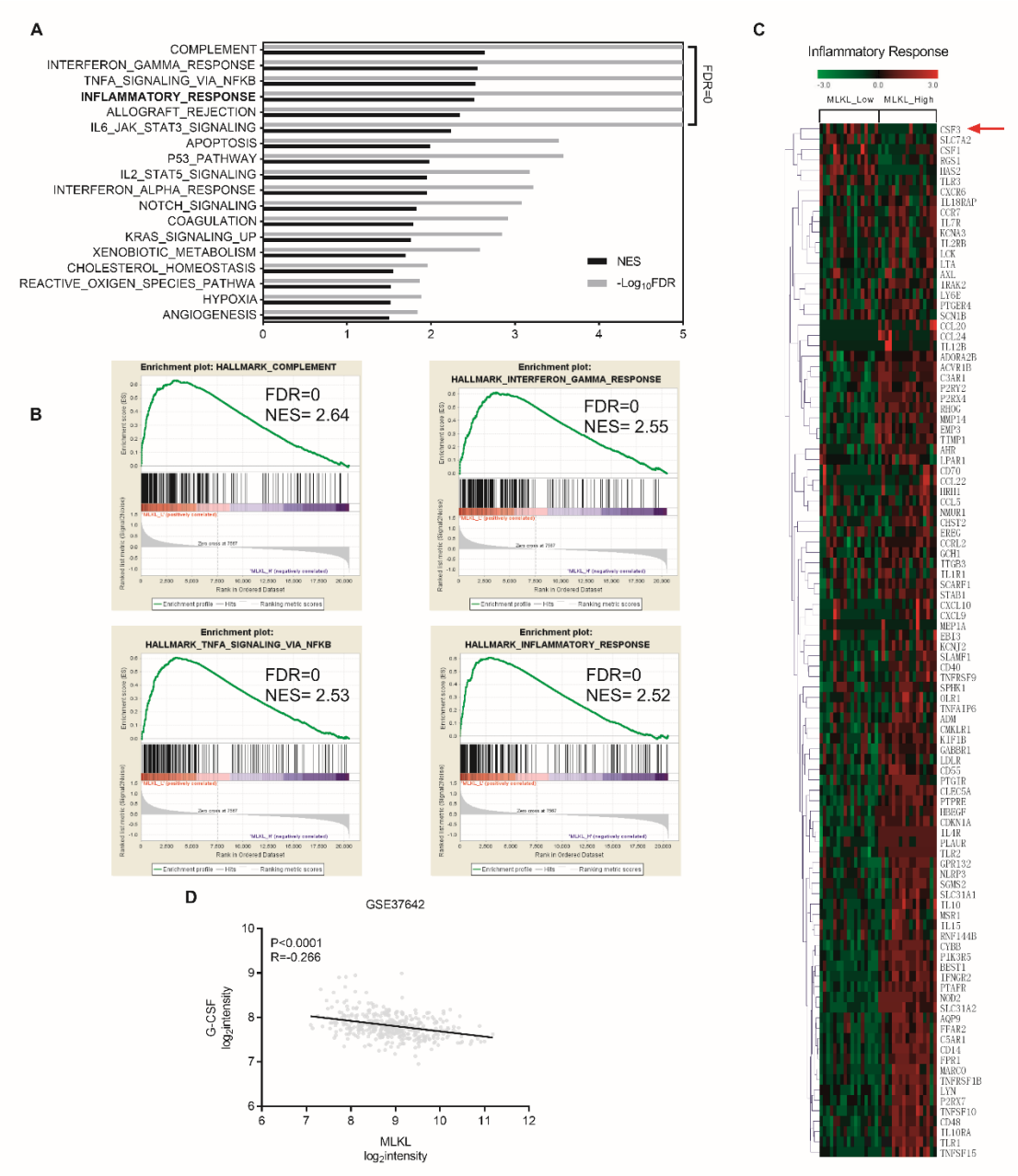

Figure 3. MLKL expression correlates with an inflammatory response signature in human AML

Shown are the top 10% MLKL high expression samples (n=17) vs. the bottom 10% MLKL low expression samples (n=17) from 173 AML patients' samples in TCGA_LAML dataset (20171013). These transcriptome data were submitted for GSEA (gene set enrichment analysis).

A, NES (normalized enrichment scores) with -log10FDR in significant pathways from GSEA analysis (the FDR of top 6 pathways are zero, thus the their -log10FDR=∞), the detailed data in Table 1.

B, the top 4 significant pathways from GSEA (gene set enrichment analysis), including complement, interferon-γ, TNF-α and inflammatory response pathways (other plots in Figure 3.1).

C, the heatmap of inflammatory response pathway, G-CSF (CSF3) expression is one of the tops differentiated between MLKL high and MLKL low samples.

D, the correlation between CSF3 and MLKL mRNA expression in GSE37642, indicating MLKL expression is negative correlated with CSF3 expression (Pearson correlation).

Of note, the analysis identified the gene expression of CSF3 (encoding for G-CSF) as one of the most differentially expressed genes in AML patients (Figure 3C). Specifically, high CSF3 (G-CSF) gene expression was observed in MLKL-low patients (Figure 3C) and vice versa (Figure 3C). To corroborate this intriguing correlation, we validated this finding in our independent gene expression AML dataset and found the same negative correlation between CSF3 and MLKL expression (Figure 3D) (GSE37642).

Collectively, our data suggested that inflammatory response gene signatures contributed to the downstream effect of the MLKL-mediated myeloid differentiation observed in AML cells, a correlation that has previously also been observed for RIPK3 (Hockendorf et al., 2016).

Pathway Name	ES	NES	FDR q-val	FWER p-val
COMPLEMENT	0.6324	2.6355	0	0
INTERFERON_GAMMA_RESPONSE	0.6137	2.5515	0	0
TNFA_SIGNALING_VIA_NFKB	0.6069	2.5288	0	0
INFLAMMATORY_RESPONSE	0.6091	2.5175	0	0
ALLOGRAFT_REJECTION	0.5611	2.3393	0	0
IL6_JAK_STAT3_SIGNALING	0.6032	2.2378	0	0
APOPTOSIS	0.4932	1.9857	0.0003	0.001
P53_PATHWAY	0.4766	1.9803	0.0003	0.001
IL2_STAT5_SIGNALING	0.4711	1.951	0.0007	0.003
INTERFERON_ALPHA_RESPONSE	0.5165	1.9497	0.0006	0.003
NOTCH_SIGNALING	0.5965	1.8301	0.0008	0.005
COAGULATION	0.4517	1.793	0.0012	0.008
KRAS_SIGNALING_UP	0.4225	1.7606	0.0014	0.01
XENOBIOTIC_METABOLISM	0.41	1.6977	0.0026	0.019
CHOLESTEROL_HOMEOSTASIS	0.4326	1.5466	0.0111	0.084
REACTIVE_OXIGEN_SPECIES_PATHWA	0.4574	1.519	0.0137	0.108

Table 4. the results of GSEA analysis

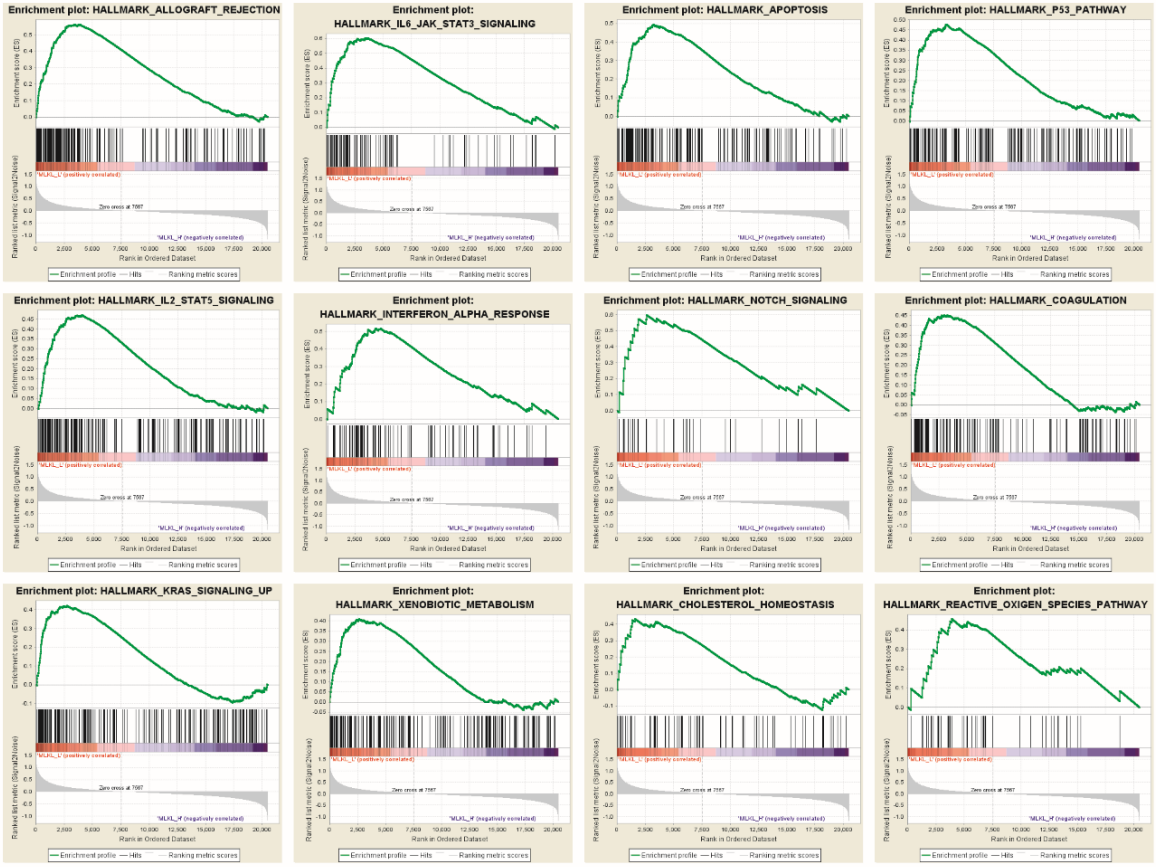

Figure 3.1. Enrichment plots for gene set enrichment analysis (GSEA) linked to Figure 3.

4.4 MLKL PROMOTES THE SECRETION OF G-CSF

G-CSF potently contributes to the proliferation and differentiation of myeloid progenitor cells (Bociek & Armitage, 1996; Skokowa et al., 2009) and changes in G-CSF levels impact on malignant myeloid progenitor levels in AML (Begley, Metcalf, & Nicola, 1987). Hence, we established an experimental system in which we measured G-CSF mRNA expression and protein levels in primary murine AML cells using primary murine progenitor cells from the bone marrow that we transduced with the oncogenic drivers AML-ETO, FLT3-ITD or MLL-ENL together with GFP (Figure 4A). After five days of culture, GFP^+ cells were sorted and subsequently cultured for 48 hours to exclude a contamination of non-transfected progenitor cells. In line with the TCGA data obtained from human AML samples (Figure 3C), we found that primary murine *Mlkl*$^{-/-}$ AML cells expressed elevated levels of CSF3 mRNA (Figure 4B). This showed that MLKL did not negatively impact on the mRNA translation of G-CSF.

In a second step, we tested for the intracellular amount of G-CSF protein in *Mlkl*$^{-/-}$ cells using an anti-G-CSF antibody that is detectable by flow cytometry upon permeabilization of the AML progenitor cells (Figure 4C). By flow cytometry we identified a significantly higher level of G-CSF in AML cells from *Mlkl*$^{-/-}$ mice when compared to WT AML cells (Figure 4C). This finding provided evidence that protein translation of CSF3 mRNA into G-CSF protein was operative and that elevated CSF3 levels resulted in increased G-CSF protein abundance in MLKL-deficient cells.

Finally, we tested for the amount of secreted soluble G-CSF in the supernatant of primary murine AML cells by flow cytometry using both WT and *Mlkl*$^{-/-}$ mice. Interestingly, the secreted amount of G-CSF was markedly higher in WT cells when compared to *Mlkl*$^{-/-}$ cells, a phenotype that was consistent across all three AML oncogenic drivers (Figure 4D). These data showed that MLKL controls the amount of secreted G-CSF in cultures of primary murine AML supportive of the notion that G-CSF-mediated cellular differentiation is impaired in the absence of MLKL.

FLT3-ITD^{+} AML cells secreted the highest levels of G-CSF, which is in line with the report showing that FLT3-ITD induces a highly inflammatory response in AML progenitor cells (Figure 4D) (Hockendorf et al., 2016). The release of alternative cytokines was not as clearly correlated to MLKL expression as that of G-CSF. We detected a reduction e.g. in GM-CSF, IL-3, IL-6 and the unconventionally secreted IL-1ß in individual AML models but not consistently across all oncogenic driver mutations (Figure 4.1).

In summary, our data provided evidence that MLKL promotes the secretion of G-CSF from transformed leukemic myeloid progenitor cells without affecting G-CSF mRNA expression and protein translation.

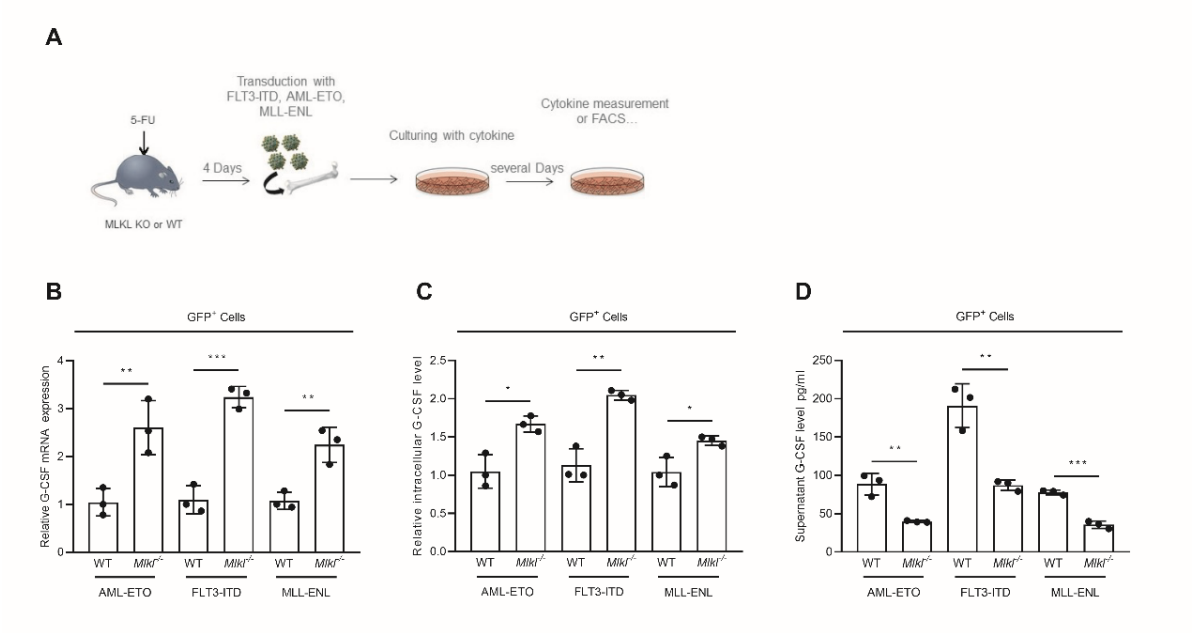

Figure 4. MLKL promotes the secretion of G-CSF.

A, we established an experimental system to generate leukemic bone marrow cells in which we transduced the oncogenic drivers AML-ETO, FLT3-ITD or MLL-ENL together with GFP to 5-FU challenged bone marrow cells.

B, bone marrow cells transduced with three different oncogenes were sorted (GFP^+) and seeded with the same cell density (2×10^6/ml). After 48 hours cells and supernatant were collected. The mRNA expression was measured by qRT-PCR, our data shown *Mlkl*$^{-/-}$ groups have higher CSF3 mRNA expression compared to the WT controls (normalized to the mean of WT group, t-test, mean with SD).

C, the same cells from B, intracellular G-CSF level was measured by FACS, we identified a significantly higher level of G-CSF in AML cells from *Mlkl*$^{-/-}$ mice when compared to WT AML cells (normalized to the mean of WT group, t-test, mean with SD), intensity plot in Figure 4.1B.

D, Supernatant G-CSF was measured by cytometric bead array, the secreted amount of G-CSF was markedly higher in WT cells when compared to *Mlkl*$^{-/-}$ cells, a phenotype that was consistent across all three AML oncogenic drivers. The intensity data in Figure 4.1B and data for other cytokines in Figure 4.1A (t-test, mean with SD).

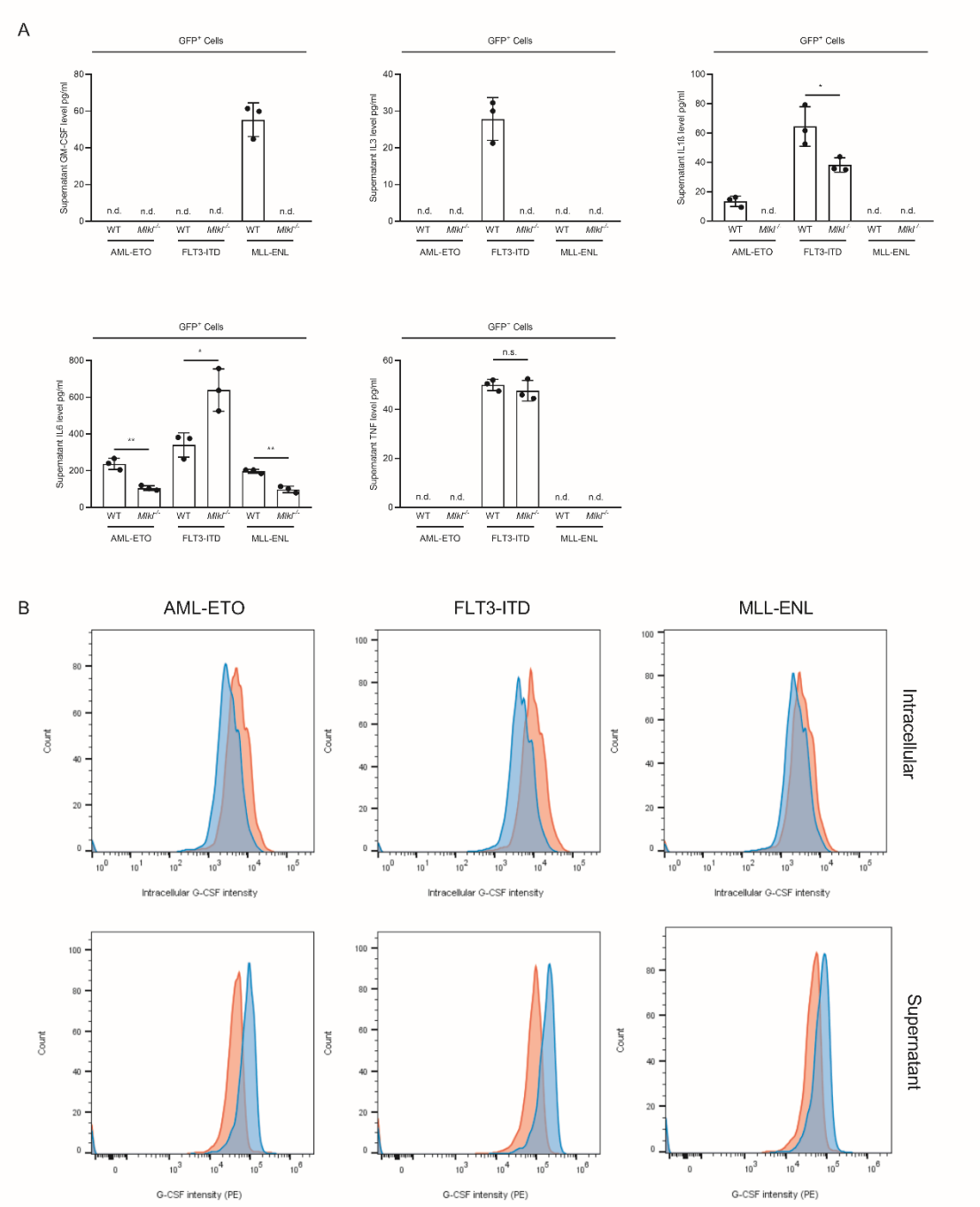

Figure 4.1. Inconsistent secretion of cytokines other than G-CSF upon loss of MLKL in AML cells.

A, The supernatant cytokine measurement for other cytokines including GM-CSF, IL3, IL1ß, IL6 and TNF. FLT3-ITD is the most inflammatory oncogenes among the three oncogenes, but the secretion pattern for other cytokines were not consistently across all oncogenic driver mutations. Same samples in Figure 4D (t-test, mean with SD).

B, the intensity plots linked to Figure 4C & D, intracellular or supernatant G-CSF was measured by cytometric bead array or intracellular FACS staining.

4.5 G-CSF RECEPTOR SIGNALING AND MYELOID DIFFERENTIATION REMAIN UNAFFECTED BY THE LOSS OF MLKL

Based on the role of MLKL is G-CSF secretion, we hypothesized that MLKL influences the myeloid progenitor differentiation in AML via its ability to control the secreted levels of G-CSF. To test this, we assayed the sensitivity of malignant myeloid progenitors for G-CSF using the different oncogenic drivers AML-ETO, FLT3-ITD or MLL-ENL. We cultured these primary murine AML cells after two days in culture with recombinant murine G-CSF (mG-CSF) to activate G-CSF receptor signaling. In addition, we cultured the AML cells with an anti-mG-CSF antibody to block G-CSF/G-CSF receptor signaling. Moreover, we treated the cells with an IgG isotype control to check for the extent of myeloid differentiation of progenitor cells in the absence of extrinsic G-CSF stimulation. As controls, non-transformed primary healthy murine bone marrow cells were treated identically and subjected to the quantification of myeloid differentiation using flow cytometry.

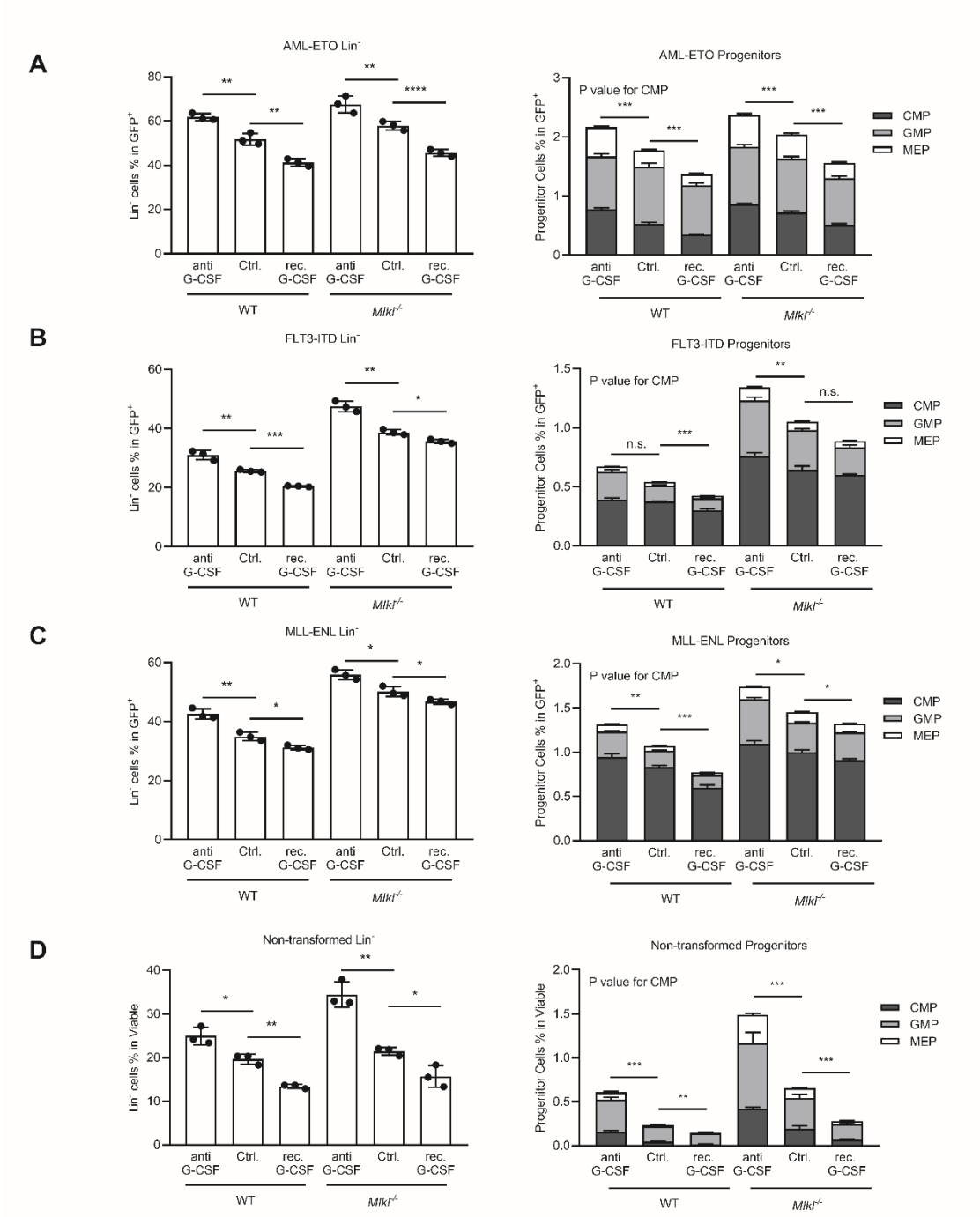

Figure 5. G-CSF receptor signaling and myeloid differentiation remain unaffected by the loss of MLKL

A, the 5-FU challenged bone marrow cells transduced with AML-ETO oncogene, the percentage of lineage negative cells (Lin^-) in GFP positive cells (GFP^+) and the percentage of progenitor cells in GFP^+ cells, we observed an expansion of Lineage negative and progenitor populations when treated with anti-G-CSF, but reduced populations when treated with G-CSF in AML-ETO oncogene condition (t-test, mean with SD).

B, same FACS staining like A, 5-FU challenged bone marrow cells transduced with FLT3-ITD oncogene, we observed an expansion of Lineage negative and progenitor populations when treated with anti-G-CSF, but reduced populations when treated with G-CSF in FLT3-ITD oncogene condition (t-test, mean with SD).

C, same FACS staining like A, 5-FU challenged bone marrow cells transduced with MLL-ENL oncogene, we observed an expansion of Lineage negative and progenitor populations when treated with anti-G-CSF, but reduced populations when treated with G-CSF in MLL-ENL oncogene condition (t-test, mean with SD).
D, 5-FU challenged bone marrow cells, the percentage of lineage negative cells (Lin^-) in viable cells and the percentage of progenitor cells in viable cells, we observed an expansion of Lineage negative and progenitor populations when treated with anti-G-CSF, but reduced populations when treated with G-CSF (t-test, mean with SD).

We found a significant expansion of myeloid progenitor cells defined either as Lineage-negative (Lin^-) cells or myeloid progenitor cells, when the cells were treated with anti-G-CSF antibody (Figure 5A-D). This finding was consistent with the notion that blocking functional G-CSF in culture prevented G-CSF-mediated myeloid differentiation.

In line with these data, we observed a reduction in myeloid progenitor cells across all three AML models when the culture was supplemented with recombined murine G-CSF in both WT and *Mlkl*$^{-/-}$ genotypes. The inflammatory milieu of FLT3-ITD$^+$ cells at baseline masked the effect of G-CSF blockade or G-CSF supplementation in regard to the amount of CMP cells, however the overall effect on the progenitor population remained remarkable (Figure 5B). Whereas the pattern of accelerated myeloid differentiation upon treatment with recombinant G-CSF and, vice versa, the repressed myeloid differentiation upon treatment with an anti-G-CSF blocking antibody remain identical between WT and *Mlkl*$^{-/-}$ AML cells, the number of myeloid progenitor cells was consistently higher in leukemic MLKL-deficient cells (Figure 5A-C). This provided evidence that G-CSF receptor signaling remained entirely functional in transformed myeloid leukemic cells upon loss of MLKL. This supported the notion that MLKL controls G-CSF secretion but not G-CSF receptor signaling to impact on AML differentiation. Of note, the same pattern as described for leukemic cells was also observed in primary murine bone marrow progenitor cells when treated with recombinant G-CSF or anti-G-CSF antibody (Figure 5D). We found that loss of MLKL did not affect healthy myeloid progenitor differentiation in response to G-CSF treatment or anti-G-CSF antibody treatment (Figure 5D). Consistent with our data in AML, even untransformed primary healthy murine bone marrow cells presented with an expansion of the primitive (Lin^-/progenitor) population (Figure 5D). This supported the notion that MLKL controls G-CSF secretion whereas G-CSF receptor signaling remained functional in *Mlkl*$^{-/-}$ cells.

The overall relatively small number of primitive progenitors obtained from healthy murine bone marrow in culture (generally less than 1%) was sensitive to G-CSF or anti-G-CSF as expected supporting the functional role of G-CSF in propagating myeloid progenitor differentiation (Figure 5D).

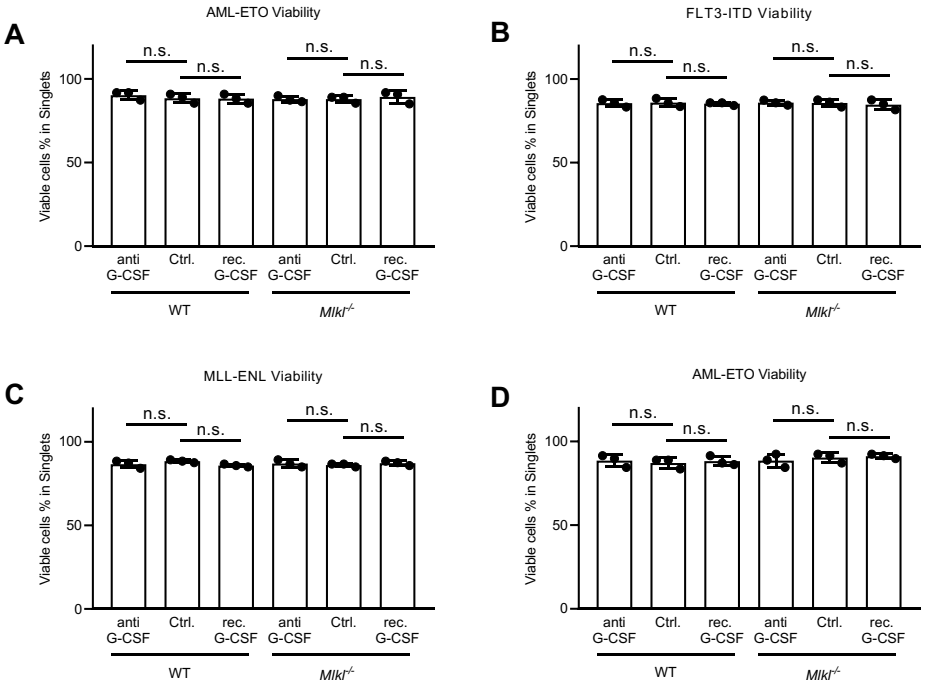

Figure 5.1. G-CSF signaling fails to affect cell death.

A-D, the 5-FU challenged bone marrow cells transduced with AML-ETO, FLT3-ITD or MLL-ENL oncogenes or without oncogenes, the viability data linked to Figure 5 A-D respectively, and we didn't see the viability difference between WT and *Mlkl*$^{-/-}$ groups, and normal bone marrow cells response better to G-CSF or anti-G-CSF treatment compared to bone marrow cells with oncogenes. (t-test, mean with SD).

In summary, *Mlkl*$^{-/-}$ myeloid progenitor cells remained sensitive to G-CSF receptor signaling and subsequent myeloid differentiation across the three AML models and in healthy untransformed myeloid progenitors.

4.6 MLKL CONTRIBUTES TO G-CSF SECRETION VIA THE ENDOSOMAL PATHWAY

It has been reported that MLKL facilitates endosomal trafficking thereby controlling the endocytosis and degradation of membrane-associated receptors such as EGF/EGFR (Yoon et al., 2017). This function of MLKL was reportedly independent of its pro-death function (Yoon et al., 2017). To explore the mechanism of how MLKL influences the secretion of G-CSF in primary murine myeloid cells, we co-stained G-CSF together with the early endosomal marker EEA1 to quantify the distribution of G-CSF and the endosomal compartment using confocal microscopy. To obtain a sufficiently high resolution of G-CSF for microscopy, the cells were stimulated with LPS prior to the analysis, which triggers G-CSF production and secretion (He et al., 2009). One day after LPS treatment, murine bone marrow cells were stained with EEA1, G-CSF and phalloidin. We observed an increased co-localization of G-CSF with EEA1 in WT cells as compared to *Mlkl*$^{-/-}$ cells (Figure 6A & B). This finding indicated that loss of MLKL prevents G-CSF to efficiently enter the endosomal compartment. As a previous paper reported MLKL deletion slowed down the trafficking of membrane associated EGFR (Yoon et al., 2017), we examined the impact of MLKL deletion on the G-CSF secretion in these cells. We tested bone marrow cells treated with LPS followed by the measurement of intracellular protein levels of G-CSF at serial time points using flow cytometry and comparing this to the secreted G-CSF levels present in the supernatant. We found an overall reduced and delayed propensity of *Mlkl*$^{-/-}$ cells to release G-CSF into the supernatant (Figure 6C). An initial delay in G-CSF secretion was followed by a significantly reduced capacity to secrete G-CSF at later time points (up to 24 hours) (Figure 6C).

This finding contrasts with the intracellular levels of G-CSF. At baseline and during the entire experiment, intracellular protein levels of G-CSF were elevated in MLKL-deficient cells compared to WT cells (Figure 6D). This build-up on G-CSF protein levels in the cells and the failure to efficiently secrete G-CSF supported the notion that a negative feedback mechanism keeping G-CSF protein production in check upon LPS stimulation was less active in *Mlkl*$^{-/-}$ cells (Figure 6D).

Together, we showed that MLKL promotes the colocalization of G-CSF to the endosomal trafficking machinery and that reduced levels of secreted G-CSF in *Mlkl*$^{-/-}$ cells coincide with

elevated intracellular G-CSF protein levels reminiscent of a failed negative feedback mechanism.

In summary, MLKL facilitates the secretion of bio-active G-CSF by controlling endosomal trafficking independent of its pro-necrotic function. Whereas G-CSF mRNA expression, protein translation and G-CSF receptor signaling remain unaffected by loss of MLKL, leukemic cells deficient for MLKL remain more primitive due to a reduced cell-autonomous G-CSF secretion, a phenotype reminiscent also in human AML patient samples.

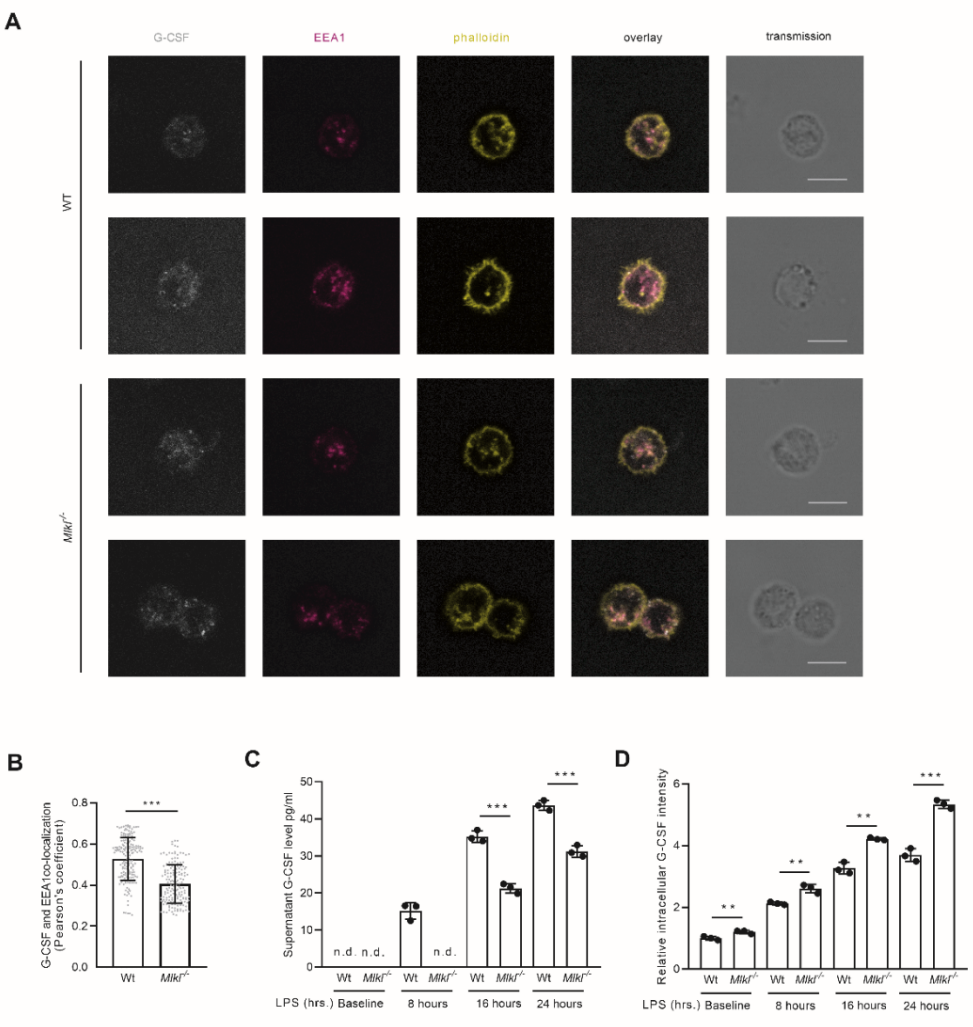

Figure 6. MLKL contributes to G-CSF secretion via the endosomal pathway

A, Immunofluorescence images, the 5-FU challenged bone marrow cells treated with 20ng/ml LPS for 48 hours (WT or Mlkl-/-) stained as follows; gray, G-CSF; magenta, EEA1; yellow, phalloidin (which stains the plasma membrane-associated F-actin), scale bar = 100μm.

B, Quantification of the immunofluorescence images, the co-localization of G-CSF and EEA1, more colocalization was observed in WT group compared to Mlkl-/- group, Pearson's coefficient (t-test, mean with SD).

C, WT or Mlkl-/- bone marrow cells treated with 20ng/ml LPS, the level of supernatant G-CSF in serial time point, We found an overall reduced and delayed propensity of Mlkl-/- cells to release G-CSF into the supernatant (t-test, mean with SD).

D, the relative intracellular G-CSF intensity in serial time point after 20ng/ml LPS treatment, G-CSF was accumulated in the Mlkl-/- bone marrow cells (normalized to the median of baseline WT group, t-test, mean with SD).

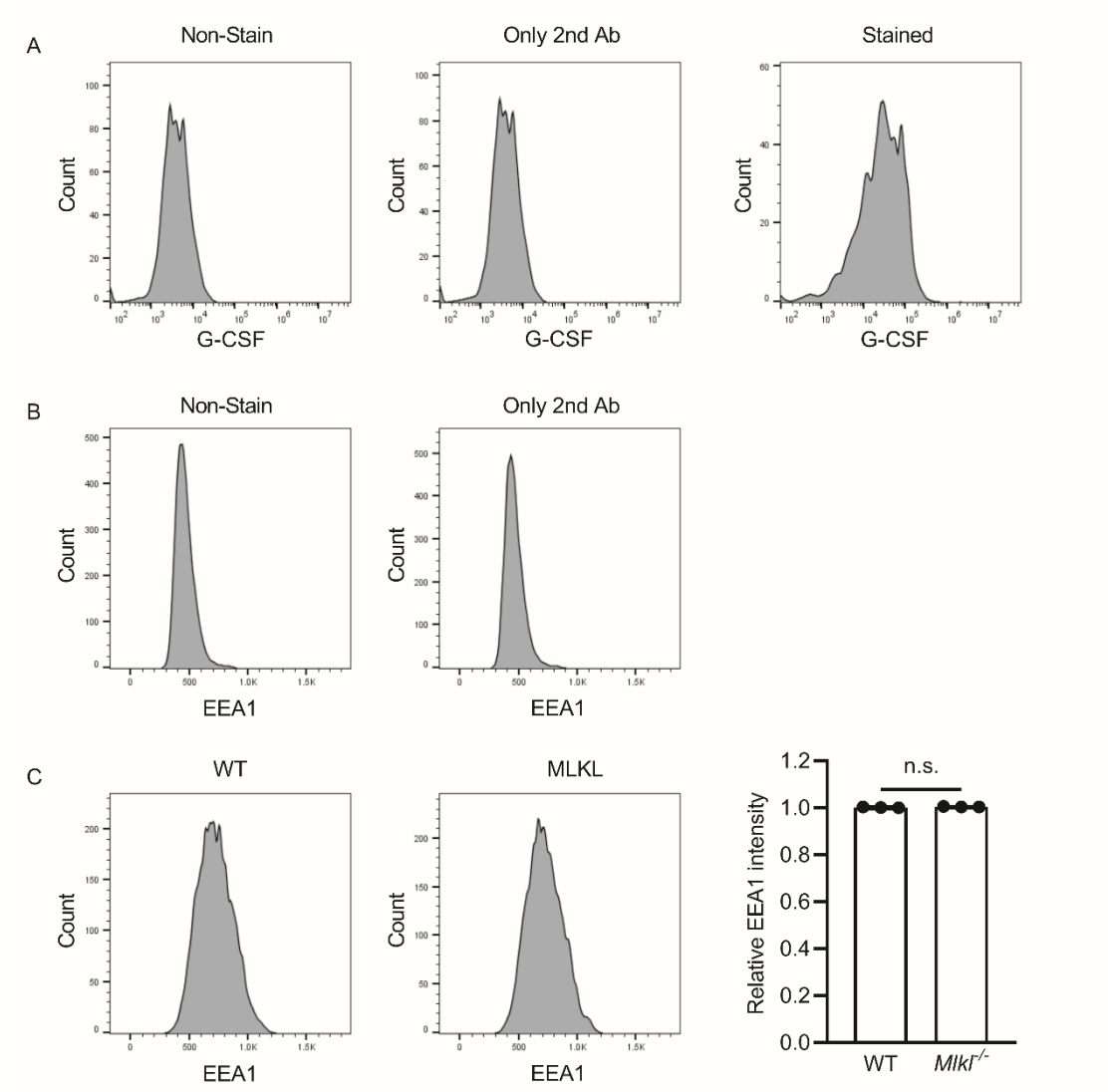

Figure 6.1. Control set-up for confocal microscopy.

A & B, negative and single 2nd antibody satin related to Figure 6A, no unspecific binding was observed between samples and 2nd antibody.

C, the relative EEA1 intensity related to Figure 6A, there is no significant difference of EEA1 between WT and Mlkl-/- (t-test, mean with SD).

MLKL serves as a critical mediator of necroptosis by inducing the formation of membrane pores upon phosphorylation by RIPK3 (Murphy et al., 2013; Sun et al., 2012; Tanzer et al., 2015). As RIPK3-dependent cell death serves as a critical tumor suppressive pathway in myeloid leukemia (Hockendorf et al., 2016), we set out to investigate the contribution of the necroptosis executioner MLKL to myeloid leukemia. In previous work, we had reported that MLKL also serves as a tumor suppressor in myeloid leukemia, however the molecular mechanism of its contribution remain unclear (Hockendorf et al., 2016). This was specifically important as *Mlkl*$^{-/-}$ primary murine leukemic cells and primary leukemic colonies remained entirely capable of undergoing cell death in response to oncogenic signaling or TNF (Hockendorf et al., 2016). This counter-intuitive result suggested that RIPK3-mediated cell death proceeded independently of MLKL eventually triggering our here presented analysis of the molecular mechanisms controlling myeloid progenitor cell integrity.

Of note, RIPK3 was sufficient and necessary to promote cellular differentiation and cell death in myeloid leukemia (Hockendorf et al., 2016). Both outcomes provide a powerful leukemia-suppressive mechanism as cellular differentiation effectively represses the clinical progression of the disease. Since cell survival remained intact in MLKL-deficient cell, we investigated cell death-independent functions of MLKL of which several have recently been reported. Xu et al. reported that MLKL regulates insulin sensitivity and participates in the development of type II diabetes (Xu et al., 2019). In addition, Ying et al. showed that MLKL is required for myelin sheath breakdown following sciatic nerve injury (Ying et al., 2018). The most relevant publication concerning the role of MLKL in endosomal traffic by Yoon and colleagues reported an endosomal trafficking function of MLKL (Yoon et al., 2017). Hence, we investigated the contribution of MLKL to endosome trafficking, cytokine secretion and leukemia development.

The “two hits and multi-hits” theory defines the cause of malignancy, which implies that more than one driver gene mutation is observed in single cancer patient (Ashley, 1969). However, in AML the exome mutation burden is lower as compared to other types of malignancy (Cancer Genome Atlas Research et al., 2013; Lawrence et al., 2014), but at least one driver

gene mutation can be identified in 96% of patients with de novo AML (Papaemmanuil et al., 2016). Thus, compared with other types of cancer the single oncogene mutation models in AML could better present the disorder. In current study three common AML driver genes were utilized, FLT3-ITD, AML-ETO and MLL-ENL. FMS Related Tyrosine Kinase (FLT3) is mutated in 20-25% of AML patients, which indicates poor prognosis; Runt-related transcription factor 1 (RUNX1) mutation is observed in 5-20% of AML patients; and MLL (KMT2A) mutation or translocation rate is around 6-8% in all AML but up to 20% in children AML and MLL mutations predict poor prognosis (Guest et al., 2016; Alexander Kohlmann, Kern, Haferlach, & Schnittger, 2013; Short et al., 2018). In our study, we demonstrated that MLKL has tumor suppressive function in acute myeloid leukemia. It promotes malignant leukemic cells differentiation which is independent of cell death. Moreover, we found that MLKL promotes the entry of G-CSF into the endosome and thereby facilitates the secretion of G-CSF outside the cells.

We have shown that mRNA expression of MLKL is reduced in human AML compared to the healthy bone marrow. The mechanism for the downregulation of MLKL expression might be regulated by copy number variation, DNA methylation, miRNA or transcription factor. Moreover, it is suppressed in medium and high-risk AML subgroups. This suggests that selection for MLKL-deficient clones exists and is likely a common feature of malignant myeloid progenitors in AML with medium and high risk. We demonstrate that MLKL signaling is a key tumor-suppressive mechanism in AML. We identify its critical function for differentiation in AML which is independent of cell death. Genetic, epigenetic and microenvironment alterations that block cellular differentiation trigger the development of acute myeloid leukemia (Behrmann, Wellbrock, & Fiedler, 2018; Fathi et al., 2018). The target differentiation therapy for acute promyelocytic leukemia (APL) serves as a successful template for subsequent development of similar treatments in other leukemias (Daenen, Vellenga, van Dobbenburgh, & Halie, 1986; Huang et al., 1988).

The microenvironment including cytokine level alteration influence the cellular differentiation in acute myeloid leukemia (Hemmati, Haque, & Gritsman, 2017; Tsapogas, Mooney, Brown, & Rolink, 2017). Therefore, we investigate the cytokine level in bone marrow cells. Our data

reports that in all three different oncogene groups, the G-CSF mRNA expression and protein level is increased inside *Mlkl*$^{-/-}$ cells as compared to the WT. However, in the supernatant G-CSF level is reduced in *Mlkl*$^{-/-}$ cells as compared to the WT. Thereby, MLKL facilitates the secretion of G-CSF outside the cells.

It has been reported that 70% of TNF-positive vesicles secreted from macrophage expresses the endosomal marker EEA1 (Chew et al., 2015). This suggest that cytokine secretion might be dependent on EEA1^{+} endosomes. As MLKL might influence the EEA1^{+} endosomes (Yoon et al., 2017). We co-stained EEA1 and G-CSF by using confocal microscopy and measured the G-CSF secretion rate on treatment with LPS. Thereby, we confirmed the distribution of G-CSF with endosomal marker EEA1. We found that EEA1 and G-CSF were more co-localized in the WT as compared to the MLKL deficient hematopoietic cells. This indicates, MLKL might promote the entry of G-CSF into the endosome. Upon stimulation with LPS, the supernatant G-CSF were measured at serial time point. We show that, MLKL deletion could slow down the endosomal trafficking which is in consensus with previous published data (Yoon et al., 2017). G-CSF can induce the differentiation in human cell lines and primary human bone marrow cells or peripheral blood mononuclear cells (Begley et al., 1987; Skokowa et al., 2009). Similar with human data, in murine progenitor cells, mG-CSF treatment can induce the differentiation of both WT and *Mlkl*$^{-/-}$ progenitor cells. This can decrease the percentage of common myeloid progenitors (CMP) which is multipotent and more primitive. This indicates, alike WT, *Mlkl*$^{-/-}$ progenitor cells remain enough sensitive to G-CSF signaling pathway irrespective of oncogene transduction. Therefore, once stimulated with G-CSF the WT and *Mlkl*$^{-/-}$ progenitors both can differentiate into less primitive cells. For further validation, we neutralized G-CSF with functional grade anti-G-CSF antibody. We found that the differentiation of progenitor cells was restricted in both WT and *Mlkl*$^{-/-}$ bone marrow cells. This led to an increase in progenitor cell population and thereby expanded CMP population as evidenced by progenitor's cell membrane antigens.

In summary, our findings provide evidence for a tumor-suppressive function of *MLKL* as it restricts myeloid leukemogenesis by facilitating G-CSF secretion which thereby promote the differentiation of hematologic progenitor cells.

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Author:

Xin Wang

° C	Degree Celsius
µl	Microliter
5-FU	5-Fluorouracil
Ab	Antibody
AML	Acute myeloid leukaemia
AML-ETO	RUNX1 - Runt related Transcription Factor 1
ANOVA	Analysis of Variance
APL	Acute promyelocytic leukemia
CD	Cluster of differentiation
CNAs	Copy Number Alterations
Ct	Threshold cycle
DAMPs	Damage-associated molecular patterns
FACS	Flow cytometry
FLT3-ITD	FMS-like tyrosine kinase 3 internal tandem duplication
G-CSF	Granulocyte-colony stimulating factor
G-CSFR	G-CSF receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
IL1 β	Interleukin-1 beta
IL3	Interleukin 3
IL6	Interleukin 6
KO	Knockout
L	Liter
LPS	Lipopolysaccharides
M-CSF	Macrophage colony-stimulating factor
miRNA	Micro RNA
ml	Milliliter
MLKL	Mixed lineage kinase domain-like protein
MLL-ENL	Mixed-lineage leukemia ENL fusion proteins
mm	Millimeter
PI	Propidium iodide
qRT-PCR	Quantitative Reverse Transcriptase PCR
RIPK1	Receptor-interacting protein 1
RIPK3	Receptor-interacting protein3
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
SCF	Stem cell factor
SD	Standard Deviation
SEM	Standard error of the mean
TNF	Tumour Necrosis Factor
TNF-R	TNF-receptor

WT	Wild type

10 FIGURE LIST

11 TABLE LIST