Acute myeloid leukemia cells polarize macrophages towards a leukemia supporting state in a Growth factor independence 1 dependent manner

Yahya S. Al-Matary,[1*] Lacramioara Botezatu,[1*] Bertram Opalka,[1] Judith M. Hönes,[1] Robert F. Lams,[1] Aniththa Thivakaran,[1] Judith Schütte,[1] Renata Köster,[1] Klaus Lennartz,[2] Thomas Schroeder,[3] Rainer Haas,[3] Ulrich Dührsen,[1] and Cyrus Khandanpour[1]

[1]Department of Hematology, University Hospital of Essen, West German Cancer Center (WTZ); [2]Institute of cell biology (Tumor Research), University Hospital Essen, University of Duisburg-Essen; [3]Department of Hematology, Oncology and Clinical Immunology, Heinrich Heine University Düsseldorf, University Hospital, Germany

**Y.S.A-M. and L.B. contributed equally to this work.*

 doi:10.3324/haematol.2016.143180

Received: January 20, 2016.

Accepted: July 07, 2016.

Pre-published: July 07, 2016.

Correspondence: cyrus.khandanpour@uk-essen.de

Supplementary Material and Methods

Transplantation experiments

Leukemia in mice was induced by transplanting lineage-negative (Lin^-) BM cells transduced with retroviruses carrying the *AML1-ETO9a* or the *MLL-AF9* oncofusion genes as well as the GFP-encoding gene as previously described.[1, 2] For primary transplantations about 5-7x10^5 *AML1-ETO9a*-transduced Lin^- BM cells or 1x10^5 *MLL-AF9*-transduced Lin^- BM cells (GFP^+) from WT mice were injected together with 1.5x10^5 competitive BM cells from *Gfi1*-WT mice into the tail vein of lethally irradiated (10 Gy) congenic recipient mice. After transplantation, the mice were monitored every second day and when moribund, the emerging BM leukemic cells were preserved in liquid nitrogen for subsequent experiments or directly used for secondary transplantations. For secondary transplantations, 1x10^5 *AML1-ETO9a* fresh GFP^+ leukemic BM cells (or 1x10^5 frozen *MLL-AF9* leukemic BM cells were transplanted into sublethally irradiated (3 Gy) *Gfi1*-WT or *Gfi1*-KO (knock-out) secondary recipient mice. In an independent experiment, we created leukemia by transplanting 1-4x10^5 C1498GFP cells[3] into sublethally irradiated (3 Gy) recipient mice.

Macrophage co-culture with leukemic cell lines

Bone marrow-derived macrophages (BMDMs) were prepared from BM cells of mice using macrophage colony stimulating factor (M-CSF), (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.[4, 5] In brief, 1.5-2x10^6 BM cells were cultured in DMEM-Glutamax (Gibco) containing 10% fetal bovine serum (FBS) (PAN^{TM} BIOTECH), 1% penicillin/streptomycin (Gibco) and 10 ng/ml M-CSF. After 7 days the macrophages were harvested using trypsin-EDTA (Gibco) and the expression of CD11b and F4/80 surface markers was determined by flow cytometry. Normally, the macrophage purity was higher than 90%. 2-3x10^5 macrophages per well were then cultured in 4 wells of 24 well plate (Falcon, New York, NY, USA). On day 1, the supernatant was discarded and 5x10^4 C1498GFP cells were added into three wells in the same medium and one well without C1498GFP cells, as a negative control. After 6

days of co-culture, the suspension cells were collected and the non-adherent cells were washed with DPBS (Gibco) and collected too. The adherent cells were detached using trypsin-EDTA (Gibco) for 5 min at room temperature. The adherent and non-adherent cells in each well were counted manually and the frequency of live C1498GFP cells was determined at a FLOWScan flow cytometer (BD Biosciences, Heidelberg, Germany). Afterwards, the number of GFP^+ was calculated. In an alternative approach, we cultured sorted GFP^- macrophages ($CD11^+Ly6G^-$) from transplanted leukemic mice as previously described.[6] In brief, $1.5x10^4$ of sorted cells were cultured per well in 48 well plates in DMEM-Glutamax containing 10% FBS and 1% penicillin/streptomycin. $5x10^4$ C1498GFP cells were added to each well. After 48 hours, the non-adherent leukemic cells in each well were harvested by collecting supernatants and detaching the remaining cells using trypsin-EDTA. The cells were then manually counted and the frequency of live GFP^+ cells was determined by a FLOWScan flow cytometer.

Macrophage polarization experiments

BMDMs were prepared using M-CSF as described above. The macrophage purity ($CD11b^+F4/80^+$) was >95%. After 6 days, supernatants were discarded and wells were washed with DPBS (Gibco). BMDMs were then polarized into M1 macrophages using 2 ml of DMEM-Glutamax (Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin and 100 ng/ml LPS (Sigma-Aldrich, Taufkirchen, Germany) or 100 ng/ml INF-y (Peprotech INC, Hamburg, Germany) and into M2 macrophages using 20 ng/ml IL4 (MiltenyiBiotec).[7, 8] After 48 hours, the M1 and M2 polarization was examined by flow cytometry and RT-PCR. The supernatants were collected, filtered and frozen for subsequent ELISA measurement of cytokine productions.

Flow cytometry and antibodies

Murine leukemic BM cells were collected by flushing femurs, tibiae and humeri with FACS buffer (DPBS containing 2% FCS and 1% penicillin/streptomycin). Human mononuclear cells were extracted from the BM of leukemic patients and healthy volunteers using Ficoll density gradient centrifugation. The cells were stained and

analysed using LSR II or FLOWScan flow cytometers (BD Biosciences). A preparative FACS Vantage SE and Diva option flow cytometer (BD Biosciences) was used for cell sorting. Raw FACS data were analysed using the FlowJo software (Tree Star, Inc., OR, USA). The following anti-mouse antibodies from Biolegend (Fell, Germany) were used: CD11bPerCP clone: M1/70, (Cat. No.101228), Ly6G PE clone: 1.A8, (Cat. No. 127607), MHC II APC clone: M5/114.15.2, (Cat. No.107613), Ly6C PE/Cy7 clone: HK1.4, (Cat. No.128017), F4/80 APC clone: MB8, (Cat. No.1231115) and CD206 PE clone: C068C2, (Cat. No.141705). For staining the human samples, the following anti-human antibodies from Biolegend were also used: PE/Cy5 CD206 clone: 15-2, (Cat. No. 321108) and APC CD163 clone: GHI161, (Cat. No. 333609). The anti-human antibody PE CD14 clone: M5E2, (Cat. No. 561707) was purchased from BD Bioscience (Heidelberg, Germany).

Quantitative RT-PCR

Total RNA was extracted from sorted macrophages ($GFP^-CD11^+Ly6G^-$) derived from transplanted mice or from sorted macrophages ($CD11b^+F4/80^+$) derived from *in vitro* co-culture experiments using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The Advantage® RT-for-PCR Kit from Clontech Laboratories (Takara, Kyoto, Japan) was used for cDNA synthesis according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed as previously described[9] using the Real-Time PCR system One Step Plus (Applied Biosystems, Thermo Fisher Scientific, Schwerte, Germany). The following TaqMan assays (Applied Biosystems) were used: *Gfi1* (Mm00515853_m1), *Arg1* (Mm00475988_m1), *IL-6* (Mm00446190_m1), *Nos2* (Mm00440502_m1) and *IL-10* (Mm01288386_m1). Gene expression was normalized to the endogenous control *Gapdh1* (Mm03302249_g1) using the $\Delta\Delta$CT method.

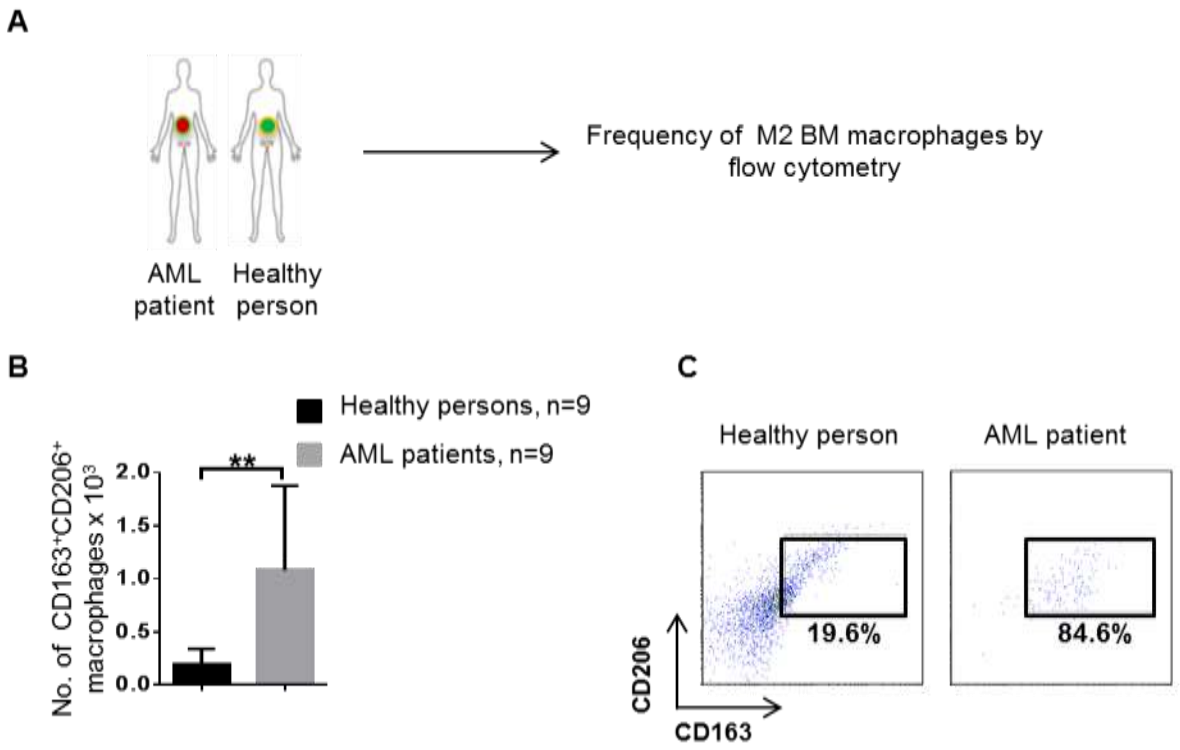
Measurement of cytokine production of macrophages

Supernatants from M1 and M2 macrophages were collected, filtered and stored in small aliquots at -80°C until further analysis. Also, $5x10^4$ sorted macrophages ($GFP^-CD11^+Ly6G^-$) derived from transplanted leukemic and non-leukemic mice were cultured

in DMEM-Glutamax containing 10% FBS and 1% penicillin/streptomycin. After 24 hours the supernatants were collected, and the level of IL-1B, IL-6 and IL-10 was measured using the mouse ELISA Ready-SET (eBioscience, Frankfurt am Main, Germany), according to the manufacturer instructions.

References

1. Krivtsov AV, Twomey D, Feng Z, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature. 2006;442(7104):818-822.
2. Yan M, Kanbe E, Peterson LF, et al. A previously unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. Nat Med. 2006;12(8):945-949.
3. Zhang L, Gajewski TF, Kline J. PD-1/PD-L1 interactions inhibit antitumor immune responses in a murine acute myeloid leukemia model. Blood. 2009;114(8):1545-1552.
4. Longbrake EE, Lai W, Ankeny DP, Popovich PG. Characterization and modeling of monocyte-derived macrophages after spinal cord injury. J Neurochem. 2007;102(4):1083-1094.
5. Choi HJ, Kim HG, Kim J, et al. Pro-apoptotic action of macrophage inhibitory cytokine 1 and counteraction of activating transcription factor 3 in carrageenan-exposed enterocytes. Toxicol Lett. 2014;231(1):1-8.
6. Chen SY, Yang X, Feng WL, et al. Organ-specific microenvironment modifies diverse functional and phenotypic characteristics of leukemia-associated macrophages in mouse T cell acute lymphoblastic leukemia. J Immunol. 2015;194(6):2919-2929.
7. Ying W, Cheruku PS, Bazer FW, Safe SH, Zhou B. Investigation of macrophage polarization using bone marrow derived macrophages. Journal of visualized experiments : JoVE. 2013;76):
8. Davis MJ, Tsang TM, Qiu Y, et al. Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in Cryptococcus neoformans infection. MBio. 2013;4(3):e00264-00213.
9. Khandanpour C, Phelan JD, Vassen L, et al. Growth factor independence 1 antagonizes a p53-induced DNA damage response pathway in lymphoblastic leukemia. Cancer Cell. 2013;23(2):200-214.



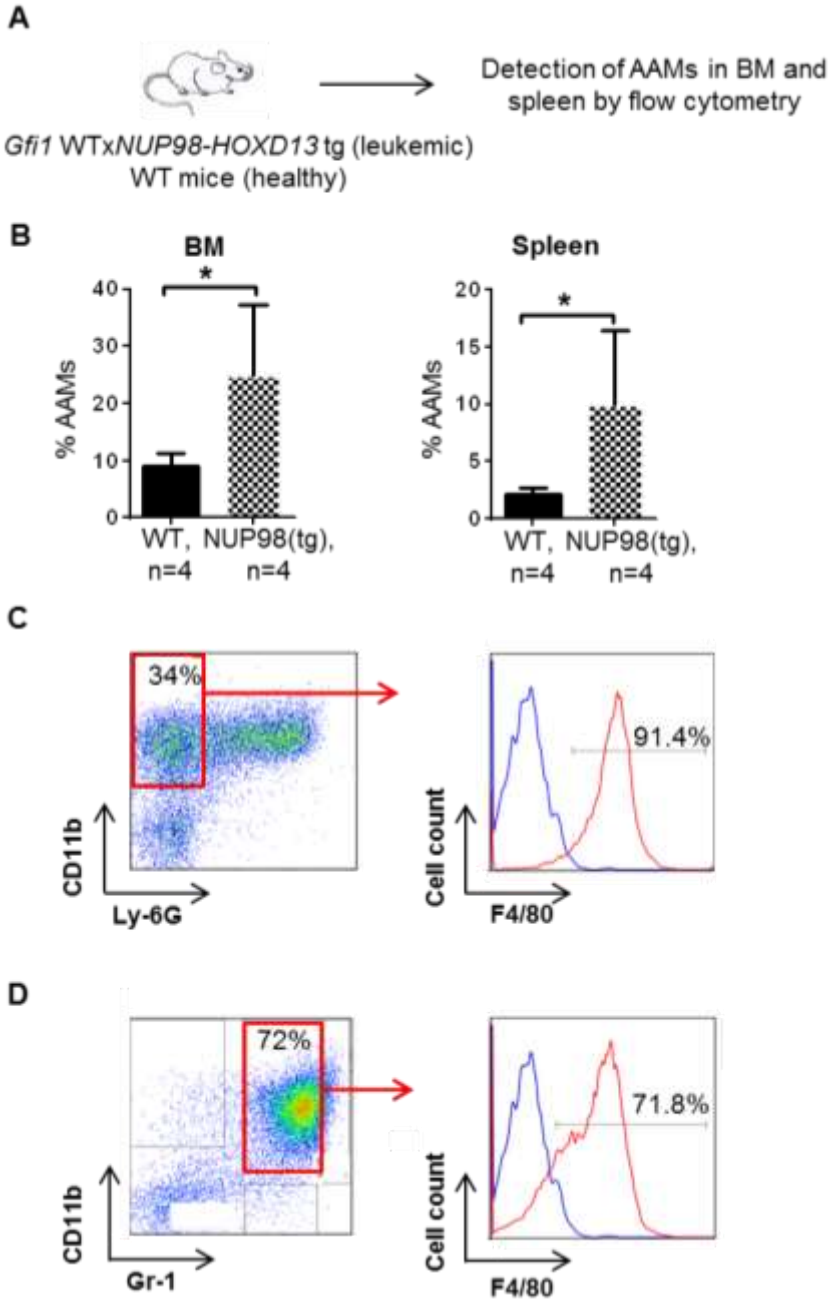
Supplementary Figure 1. Accumulation of $CD163^{+}CD206^{+}$ M2 like macrophages in BM of AML patients.

A) Schematic illustration of the experimental design. BM mononuclear cells from pre-diagnosed AML patients and healthy volunteers were isolated by Ficoll density gradient centrifugation and the frequency of M2 macrophages was evaluated using flow cytometry analysis.

B) The absolute numbers of BM $CD163^{+}CD206^{+}$ M2 macrophages gated on CD14-expressing macrophage/monocyte cell population (out of 100000 cells acquired by FACS) assessed in 9 AML patients and 9 healthy volunteers, (**p=0.008).

C) Representative FACS plots from BM of one AML patient and one healthy volunteer demonstrating the frequency of $CD163^{+}CD206^{+}$ M2 macrophages gated within total $CD14^{+}$ monocytes.

Abbreviations: BM=bone marrow, AML=acute myeloid leukemia, FACS=fluorescence-activated cell sorting.



Supplementary Figure 2. Increased frequency of AAMs in BM and spleen of leukemic *NUP98-HOXD13* mice.

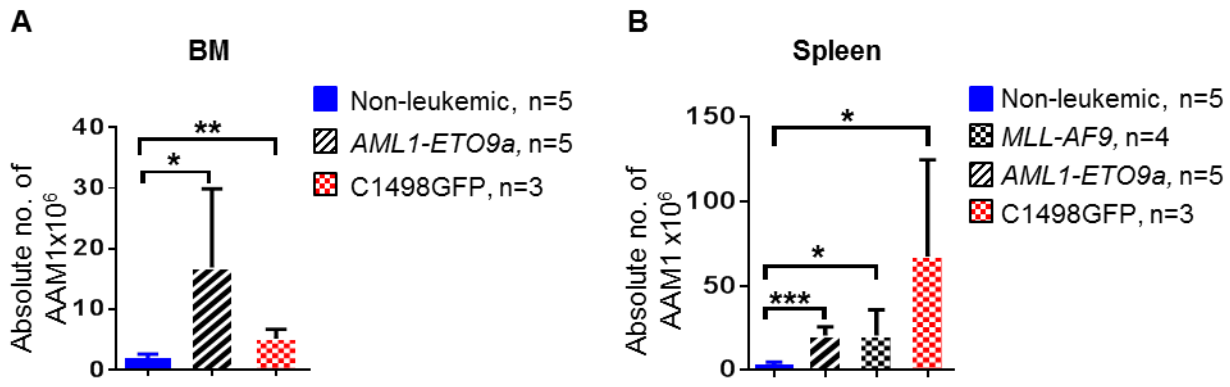
A) Schematic representation of the experimental design. Leukemic *Gfi1*-WTx*NUP98HOXD13* and healthy WT mice were sacrificed and the frequency of AAMs was determined by flow cytometry.

B) The frequency *of* $CD11b^{+}Ly6G^{-}$ monocytes/macrophages in BM (left panel) and spleen (right panel) of leukemic *Gfi1*-WT*xNUP98-HOXD13* (n=4) and non-leukemic WT mice (n=4) (*p=0.05 for BM and *p=0.04 for the spleen).

C) Representative FACS plots showing that more than 90% of BM $CD11b^{+}Ly6G^{-}$ monocytes/macrophages are positive for the F4/80 surface marker.

D) Representative FACS plots showing that the majority of $CD11b^{hi}Gr1^{int}$ monocytes/macrophages in BM are also positive for the F4/80 surface marker.

Abbreviations: BM=bone marrow, AAMs=acute myeloid leukemia associated macrophages, FACS=fluorescence-activated cell sorting, WT=wild type.

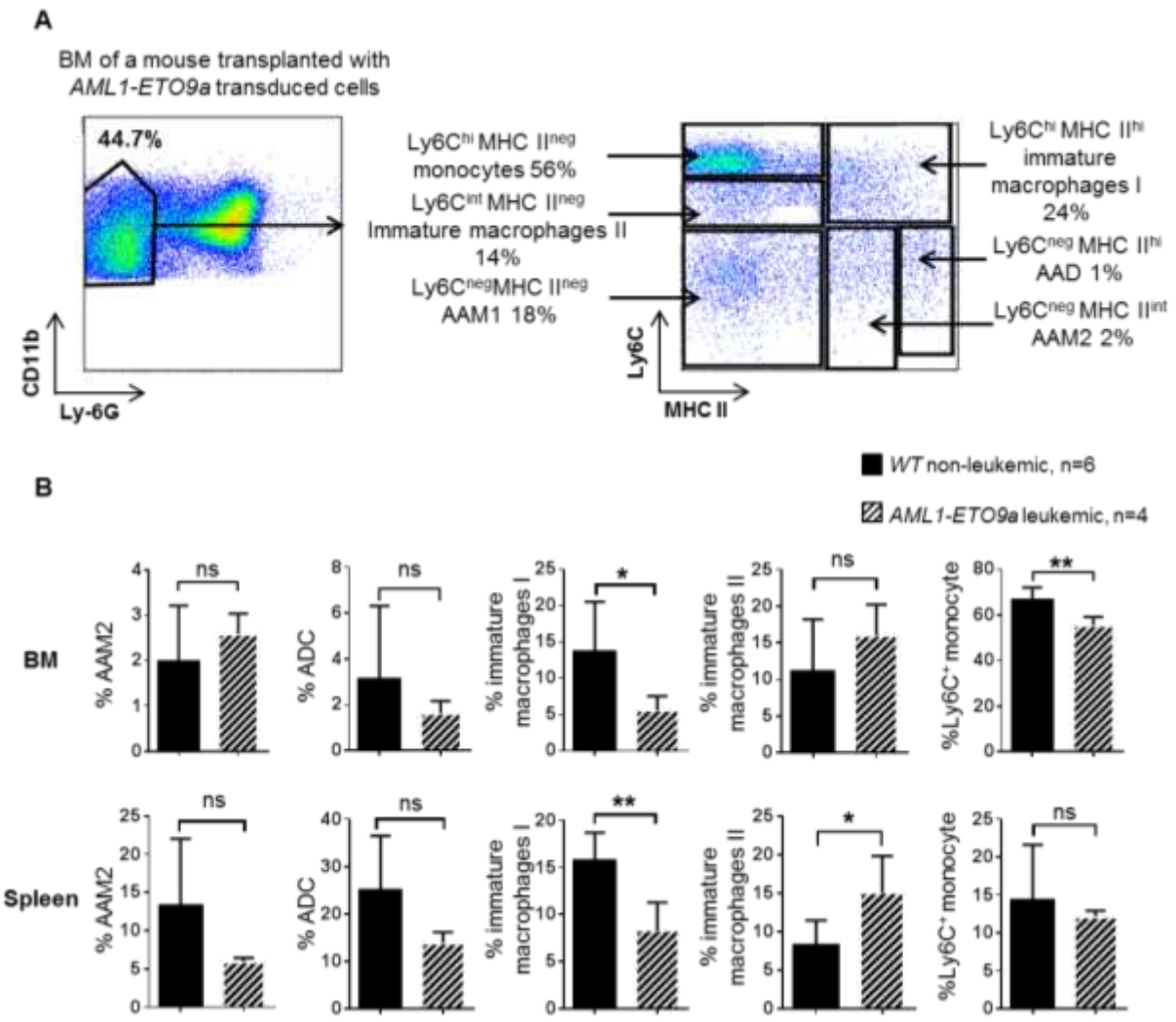


Supplementary Figure 3. The absolute number of AAM1 is significantly increased in BM and spleen of leukemic transplanted mice

A) The absolute numbers of Ly6C$^-$MHC II$^-$ AAM1 assessed in BM of leukemic mice transplanted either with *AML-ETO9a* transduced cells (secondary recipients, n=5) or C1498GFP cell line (n=3) compared to control non-leukemic mice transplanted with non-transduced cells (n=5), (**p=0.008 for C1498GFP, *p=0.03 for *AML1-ETO9a)*.

B) The absolute numbers of Ly6C$^-$MHC II$^-$ AAM1 assessed in spleen of leukemic mice transplanted with *AML-ETO9a* (n=5), *MLL-AF9* (n=3) or C1498GFP cell line (n=3) compared to control non-leukemic mice transplanted with non-transduced cells (n=5), (*p=0.02 for *MLL-AF9,* *p=0.02 for C1498GFP, ***p<0.0001 for *AML1-ETO9a)*.

Abbreviations: BM=bone marrow, AAM1=acute myeloid leukemia-associated macrophages type 1

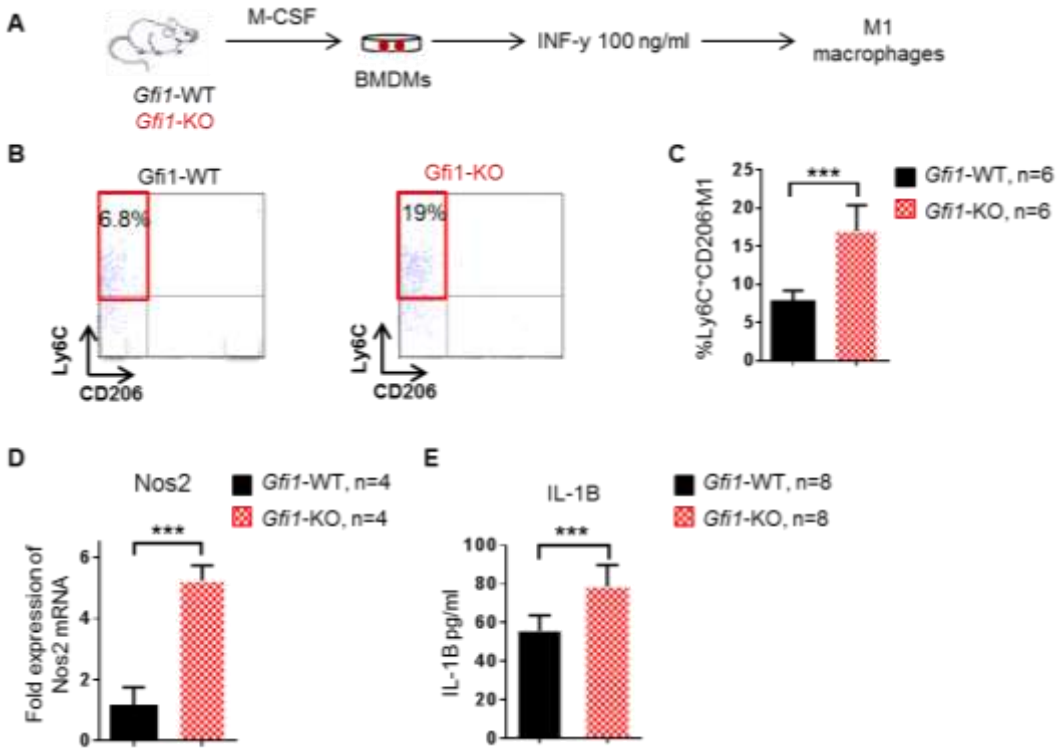


Supplementary Figure 4. The distribution of different macrophage classes in BM and spleen of *AML1-ETO9a* leukemic mice.

A) Leukemia-associated monocytes ($GFP^-CD11b^+Ly6G^-$) were classified into 6 classes according to the surface expression of Ly6C and MHCII markers. Representative FACS profiles showing the gating strategy for the 6 macrophages classes in leukemic mice transplanted with *AML1-ETO9a* transduced cells.

B) The frequency of different macrophage classes in BM (upper panels) and spleen (lower panels) of leukemic mice transplanted with *AML1-ETO9a* transduced cells (n=4) or non-transduced cells (n=6) (*p=0.05 for immature macrophages I in BM, **p=0.008 for $Ly6C^+$ monocytes in BM, **p=0.004 for immature macrophages I in spleen, *p=0.02 for immature macrophage II in spleen).

Abbreviations: AML=acute myeloid leukemia, AAM1=AML-associated macrophages type 1, AAM2=AML-associated macrophages type 2, AAD=AML-associated dendritic cells.



Supplementary Figure 5. *Gfi1* affects macrophage polarization by inhibiting M1 response in INF-y-treated macrophages.

A) Schematic representation of the *in vitro* polarization experiment. *Gfi1*-WT or *Gfi1*-KO BMDMs were stimulated with INF-y (100 ng/ml) for 48 hours. Medium was collected for ELISA and M1 macrophages were characterized by flow cytometric and gene expression analysis.

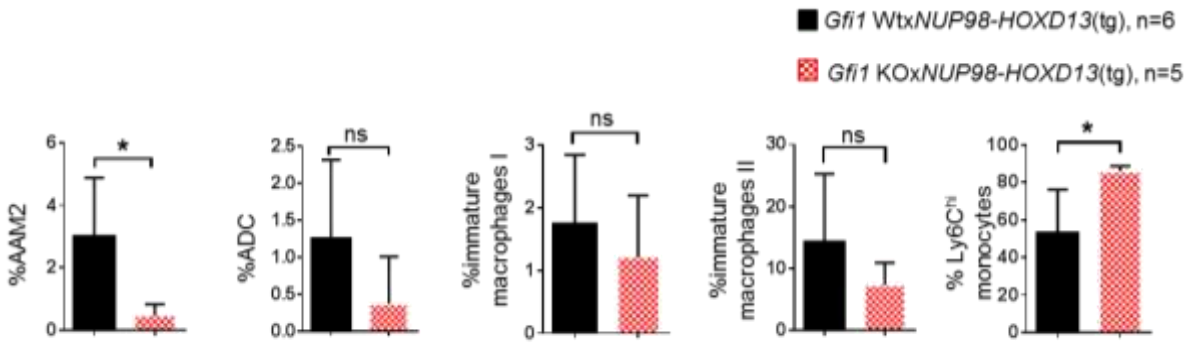
B) Representative FACS plots demonstrating the frequency of $Ly6C^{+}CD206^{-}$ M1 in BMDMs from *Gfi1*-WT and *Gfi1*-KO mice after INF-y stimulation.

C) The frequency of $Ly6C^{+}CD206^{-}$ M1 macrophages in BMDMs from *Gfi1*-WT (n=6) and *Gfi1*-KO (n=6) mice after INF-y stimulation, (***p=0.0001).

D) Fold change of Nos2 mRNA level in *Gfi1*-WT (n=4) and *Gfi1*-KO (n=4) M1 $Ly6C^{+}$ $CD206^{-}$ macrophages after INF-y stimulation. RT-PCR results of duplicates from two independent experiments are shown (***p<0.0001).

E) The level of IL-1B in supernatants of M1 macrophages derived from Gfi1-WT (n=8) and Gfi1-KO (n=8) mice was measured using ELISA commercial kits. Results of duplicates from two independent experiments are shown ($p<0.0004$).

Abbreviations: BMDMs=bone marrow derived macrophages, INF-y=Interferon-y, Nos2=nitric oxide synthase 2, IL-1B=interleukin 1B; GAPDH=Glyceraldehyde 3-phosphate dehydrogenase, FACS=fluorescence-activated cell sorting, ELISA=enzyme-linked immunosorbent assay



Supplementary Figure 6. The effect of Gfi1 on the distribution of different macrophage classes in the BM of *NUP98-HOXD13* leukemic mice.

The frequency of different macrophages classes in BM of *Gfi1*-WTx*NUP98-HOXD13* (n=6) and *Gfi1*-KOx*NUP98-HOXD13* (n=5) leukemic mice evaluated using flow cytometry analysis (*p=0.02 for AAM2 and *p=0.16 for $Ly6C^+$ monocytes).

Abbreviations: BM= bone marrow

Supplementary Table1: Characteristics of patient samples used for macrophage analysis

Patient No.	Age in years	Sex	Diagnosis	Cytogenetic	Mutations
1	32	M	AML	t(2;3)(p15-22;q26)	-
2	54	M	AML	t(15;17)(q24;q21)	PML/RARA
3	60	F	AML	45,XX,-7q	-
4	65	M	AML	Normal	-
5	59	M	AML	t(15;17) (q24;q21)	PML/RARA
6	38	F	AML	Not available	
7	36	F	AML	Not available	
8	74	M	AML	Normal	-
10	31	M	Normal	-	-
11	30	F	Normal	-	-
12	51	F	Normal	-	-
13	75	M	Normal	-	-
14	70	F	Normal	-	-
15	66	M	Normal	-	-
16	66	M	Normal	-	-
17	70	M	Normal	-	-
18	74	M	Normal	-	-

Abbreviations: F=female, M= male, AML=acute myeloid leukemia