Bone marrow endothelial cell-derived interleukin-4 contributes to thrombocytopenia in acute myeloid leukemia

Ai Gao,1,2,# Yuemin Gong,1,2,3# Caiying Zhu,1,2 Wanzhu Yang,1,2 Qing Li,1,2 Mei Zhao,1,2 Shihui Ma,1,2 Jianyong Li,3 Sha Hao,1,2,4,5,* Hui Cheng1,2,4,5,* and Tao Cheng1,2,4,5,*

1State Key Laboratory of Experimental Hematology; 2Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin; 3Department of Hematology, the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, Jiangsu; 4Center for Stem Cell Medicine, Chinese Academy of Medical Sciences, Tianjin and 5Department of Stem Cell & Regenerative Medicine, Peking Union Medical College, Tianjin, China

#These authors contributed equally to this work.

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Correspondence: SHA HAO - haoshaihcams.ac.cn
HUI CHENG - chenghui@ihcams.ac.cn
TAO CHENG - chengtao@ihcams.ac.cn
Supplementary Figure Legends

Supplementary Figure S1. Decreased platelets and megakaryocytes in AML mice.

a. Schematic diagram of the experimental design to establish the MLL-AF9 leukemia murine model. BM c-kit$^+$ cells (CD45.1) were infected with the MSCV-MLL/AF9-PGK-puro retrovirus and then transplanted into lethally irradiated mice (CD45.2). Established CD45.1$^+$ MLL-AF9 leukemia cells were serially propagated by transplanting $1 \times 10^6$ spleen cells from AML mice (day 14) into non-irradiated B6-Ly5.2 recipient mice for subsequent experiments.

b. Platelet counts in peripheral blood at indicated time points of AML. n=5 mice for each time point, three independent experiments.

c. Total number per limb of MKs in bone marrow at indicated time points of AML. n=5 mice for each time point, three independent experiments.

d. Representative flow cytometric plots of megakaryocytes (MKs) from healthy control and AML (Day 14) mice bone marrow.

Significant difference: ** p<0.01, *** p<0.001. Error bars show SEM.

Supplementary Figure S2. Representative flow cytometric plots of HSPCs from healthy control and AML (Day 14) mice bone marrow.

Supplementary Figure S3. Intact survival, cell cycle state and megakaryocytic potential of PreMegE and MkPs from AML bone marrow.

a. Percentage of Annexin V+ apoptotic cells in PreMegE subset at indicated time points of AML. n=5 for each time point, three independent experiments.

b. Percentages of cells in different phase of cell cycle in PreMegE subset at indicated time points of AML. n=5 for each time point, three independent experiments.

c-d. Representative flow cytometric plots (c) and total number (d) of CD150$^{+}$/high CD41$^{+}$/high megakaryocytic lineage formed by 1000 PreMegE cells which were isolated from control or AML (Day 14) bone marrow and cultured in vitro with
mSCF (50ng/ml), mTPO (50ng/ml), mIL-3 (20ng/ml) and EPO (2U/ml) for 3 days. 
n=5, three independent experiments.

e-f. Representative flow cytometric plots (e) and percentage of Annexin V+
apoptotic cells (f) in MkP subset at indicated time points of AML. n=5 for each time 
point, three independent experiments.

g. Percentages of cells in different phases of cell cycle in MkP subset at indicated 
time points of AML. n=5 for each time point, three independent experiments.

h. Number of megakaryocyte colonies (CFU-MKs) formed from 2000 MkPs 
isolated from control or AML (Day14) bone marrow. n=4 mice per group, two 
independent experiments

Significant difference: ** p<0.01, *** p<0.001. ns, no significant difference. Error 
bars show SEM.

**Supplementary Figure S4. Differential gene expression of MKs from control 
and AML mice.**
a. Volcano plot of differentially expressed genes between control and AML MKs. 
The X-axis represents the log2 fold change of gene expression levels. The Y-axis 
represents the –log10 P-value. Significantly upregulated genes are represented 
as ‘red’ dots and significant downregulated genes are represented as ‘green’ dots.
b. Heatmap of cytokine receptor expression in control and AML MKs. The color 
scale indicates expression values.

**Supplementary Figure S5. TPO protein levels in serum of control and AML 
mice based on ELISA measurements.**

**Supplementary Figure S6. Blocked polyploidization of megakaryocytes in 
AML bone marrow.**
a. Proportions in normal hematopoietic cells of MKs with different ploidy in mice 
bone marrow at indicated time points of AML. Data were normalized to those 
values of healthy control and presented as the folds of control. n=4-5 for each 
time point, three independent experiments.
b. Representative flow cytometric plot of MK ploidy distribution in healthy control and AML (Day14) mice bone marrow. Significant difference: * p<0.05, ** p<0.01. Error bars show SEM.

Supplementary Figure S7. Inhibition of IL-4 or induction chemotherapy alone could not result in platelet recovery in AML.

a. Schematic diagram of the experimental design: CD45.1+ leukemia cells were injected intravenously into mice on day 1. 10 mg/kg/day of anti-mIL-4 or PBS was administrated intraperitoneally on days 7, 9 and 11. Mice were sacrificed on day 13 for analysis.

b. Counts of platelets, erythrocytes and leukocytes in peripheral blood of mice injected with PBS or anti-mIL-4. n=5-6 mice per group, three independent experiments.

c. Percentage of CD45.1+ leukemia cells engraftment measured via bone marrow treated with PBS or anti-mIL-4. n=5-6 mice per group, three independent experiments.

d. Schematic of the experiment: CD45.1+ leukemia cells were injected intravenously into mice on day 1. 60 mg/kg/day of Arac was administrated intraperitoneally for one week from day 15. PB analysis was performed one day after the last administration of AraC.

e. Percentage of CD45.1+ leukemia cells engraftment measured via PB before (day 15) and after (day 22) chemotherapy. n=5 mice per group, three independent experiments.

f. Survival curves of AML mice treated with Arac or not. n=5 mice per group, three independent experiments.

g. Counts of platelets in peripheral blood of mice injected with AraC or not. n=5 mice per group, three independent experiments.

h. IL-4 protein levels in BM plasma of healthy control, AML control (Day 14) mice and AML mice receiving chemotherapy based on ELISA measurements. n=5 mice per group, three independent experiments.

i. TPO protein levels in serum of AML mice injected with AraC or combined with
anti-mIL-4. n=5 mice per group, three independent experiments.

Significant difference: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. ns, no significant difference. Error bars show SEM.
**Supplementary Table S1. Cell surface markers for phenotypical analyses of hematopoietic cells and niche cells by flow cytometry**

**Cell surface markers for flow cytometric analyses**

| Cell type | Surface markers                          |
|-----------|-----------------------------------------|
| LT-HSC    | Lin- c-Kit+ Sca1+ CD150+CD48-           |
| MPP2      | Lin- c-Kit+ Sca1+ CD150+CD48+           |
| PreMegE   | Lin- c-Kit+ Sca1+CD105-CD150+CD41-      |
| CMP       | Lin- c-Kit+ Sca1- CD16/32- CD34+        |
| MEP       | Lin- c-Kit+ Sca1- CD16/32- CD34-        |
| MkP       | Lin- c-Kit+ Sca1- CD150+ CD41+          |
| MK        | SSChigh CD41high                        |
| EC        | CD45- Ter119- CD31+                     |
| MSC       | CD45- Ter119- CD31- CD51+ Sca1+         |
| OBC       | CD45- Ter119- CD31- CD51+ Sca1-         |
Supplementary Table S2. Antibodies used for flow cytometry

Antibodies used for flow cytometry

| Antibody conjugate         | Clone       | Supplier      |
|---------------------------|-------------|---------------|
| B220 PE-CY7               | RA3-6B2     | eBioscience   |
| B220 Biotin               | RA3-6B2     | eBioscience   |
| CD3ε Biotin               | 145-2C11    | eBioscience   |
| CD3ε PE-CY7               | 145-2C11    | eBioscience   |
| CD4 Biotin                | RM4-5       | eBioscience   |
| CD4 PE-CY7                | RM4-5       | eBioscience   |
| CD8α Biotin               | 53-6.7      | eBioscience   |
| CD8α PE-CY7               | 53-6.7      | eBioscience   |
| Ter-119 Biotin            | Ter119      | eBioscience   |
| Ter-119 PE-CY7            | Ter119      | eBioscience   |
| Mac-1 Biotin              | M1/70       | eBioscience   |
| Mac-1 PE-CY7              | M1/70       | eBioscience   |
| Mac-1 APC-eFluor 780      | M1/70       | eBioscience   |
| Gr-1 (Ly-6G) Biotin       | RB6-8C5     | eBioscience   |
| Gr-1 (Ly-6G) PE-CY7       | RB6-8C5     | eBioscience   |
| Gr-1 (Ly-6G) APC-eFluor 780| RB6-8C5    | eBioscience   |
| Streptavidin APC-eFluor 780| -          | eBioscience   |
| Streptavidin BrilliantViolet 421 | -      | BioLegend     |
| c-Kit (CD117) APC         | 2B8         | eBioscience   |
| CD34 Biotin               | RAM34       | eBioscience   |
| CD34 FITC                 | RAM34       | eBioscience   |
| CD41 APC                  | MWReg30     | eBioscience   |
| CD41 PerCP-eFluor 710     | MWReg30     | eBioscience   |
| Flt3 (CD135) PE           | A2F10       | eBioscience   |
| Sca-1 PE-Cy7              | D7          | eBioscience   |
| Sca-1 APC-Cy7             | D7          | BioLegend     |
| CD124 BrilliantViolet 421 | mIL-4R-M1   | BD Bioscience |
| CD16/32 PE                | 93          | eBioscience   |
| CD150 PE                  | TC15-12F12.2| BioLegend     |
| Antibody                                      | Clone/Name | Supplier |
|-----------------------------------------------|------------|----------|
| CD45.1 PE-CY7                                 | A20        | eBioscience |
| CD45 FITC                                     | 30-F11     | eBioscience |
| CD51 PE                                       | RMV-7      | eBioscience |
| CD31 APC                                      | 390        | eBioscience |
| Annexin V FITC                                | -          | BD Bioscience |
| CD105 biotin                                  | MJ7/18     | eBioscience |
| Anti-Von Willebrand Factor antibody           | ab6994     | abcam    |
| Ki67 FITC                                     | 7B11       | eBioscience |
| Ki67 PE                                       | B56        | BD Bioscience |
| Donkey anti-Rabbit AF488                      | -          | Invitrogen |
| Stat6 (pY641) Alexa Fluor-647                 | -          | BD Bioscience |
Supplementary Figure S1. Decreased platelets and megakaryocytes in AML mice.

(a) MSCV-MLL/AF9-PGK-puro

B6.SJL (CD45.1) BM c-Kit+ cells

puromycin selection

Preleukemic cells transplantation

9.5Gy IR

CD45.1+ leukemic cells from spleen

C57BL/6J (CD45.2)

(b) Platelet count (10^9/L)

(c) Total number of MKs (10^3 per limb)

(d) MKs

SSC-A

CD41

Ctrl

AML

MKs

MKs

0.044%

0.018%
Supplementary Figure S2. Representative flow cytometric plots of HSPCs from healthy control and AML (Day 14) mice bone marrow.
Supplementary Figure S3. Intact survival, cell cycle state and megakaryocytic potential of PreMegE and MkPs from AML bone marrow.

(a) Annexin V+ cells (%) in PreMegE cells over time. (b) Percentage of cells in S/G2/M, G1, and G0 phases of the cell cycle over time. (c) Flow cytometry plots showing CD150 and CD41 expression in Ctrl and AML samples. (d) Megakaryocytes per 1000 cells. (e) CFU-Mk potential over time. (f) Annexin V+ cells (%) in MkPs. (g) Percentage of MkPs in S/G2/M, G1, and G0 phases of the cell cycle. (h) CFU-Mk potential in Ctrl and AML samples.
Supplementary Figure S4. Differential gene expression of MKs from control and AML mice.

AML MK vs Ctrl MK Volcano Plot

Regulation
- up (1156 genes)
- none
- down (1874 genes)
Supplementary Figure S5. TPO protein levels in serum of control and AML mice based on ELISA measurements.

![Bar graph showing TPO protein levels in serum of control (Ctrl) and AML mice.](image_url)
Supplementary Figure S6. Blocked polyploidization of megakaryocytes in AML bone marrow.
Supplementary Figure S7. Inhibition of IL-4 or induction chemotherapy alone could not result in platelet recovery in AML.

a) CD45.1+ leukemia cells

PBS or anti-mIL-4 Analysis

Day 1 7 9 11 13

b) PB

S

t

IL

4

0 50 100 150

ns

Ctrl  PBS  anti-IL-4

Plt (10^9/L)

RBC (10^12/L)

WBC (10^9/L)

Ctrl  PBS  anti-IL-4

c) CD45.1+ % in BM

PBS  anti-IL4

D

Gal

ay

 tolerated

D

ay

 tolerated

d) CD45.1+ leukemia cells

AraC

Analysis

Day 1 15 21 22

E

Before chemotherapy

After chemotherapy

f) Percent survival

Days elapsed

0 10 20 30

0 50 100

AML Ctrl

AML AraC

g) PB

S

t

IL

4

0 50 100 150

ns

Ctrl  PBS  anti-IL-4

Plt (10^9/L)

RBC (10^12/L)

WBC (10^9/L)

Ctrl  PBS  anti-IL-4

h) IL-4 concentration in BM plasma (pg/ml)

Ctrl  AML  Arac

ns

i) TPO concentration in serum (pg/ml)

AraC  AraC+anti-IL-4

ns